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Abbreviations

ART	Artificial reproductive technology
AUCROC	Area under receiver operating curve
CCND2	Cyclin D2
CP	Cerebral palsy
cSET	Compulsory single embryo transfer (SET when only one embryo is available for transfer)
CTNND1	Catenin delta-1
CXCR4	CXC chemokine receptor 4
DET	Double embryo transfer
DHCR7	7-dehydrocholesterol reductase
DVL3	Dishevelled dsh homolog 3
DZ	Dizygotic
EC	Early cleavage
eSET	Elective single embryo transfer (SET when more than one embryo is available for transfer)
ET	Embryo transfer
FISH	Fluorescent in situ hybridization
GP	General practitioner
GPX3	Glutathione peroxidase 3
GQE	Good quality embryo
hCG	Human chorionic gonadotropin
HSPB1	Heatshock 27 kDa protein 1
ICER	Incremental cost-effectiveness ratio
ICSI	Intracytoplasmic sperm injection
IUI	Intra-uterine insemination
IVF	In vitro fertilization
MNB	Multinucleated blastomere
MZ	Monozygotic
NEC	Non-early cleavage
NICU	Neonatal intensive care unit
NPB	Nucleolar precursor body
OHSS	Ovarian hyperstimulation syndrome
OPU	Ovum pick up
OR	Odds ratio
PGD	Preimplantation genetic diagnosis
PGS-AS	Preimplantation genetic screening – aneuploidy screening
PN	Pronucleus
PR	Pregnancy rate
qRT-PCR	Quantitative real-time polymerase chain reaction
RCT	Randomized controlled trial
ROS	Reactive oxygen species
SET	Single embryo transfer
SP	Singleton pregnancy
TLDA	Taqman low density array
TP	Twin pregnancy
TRIM28	Tripartite motif-containing 28

CHAPTER 1

General Introduction

Preface

Human reproduction can be considered very inefficient. Per menstrual cycle, around 30% of the conceptions is lost before implantation, another 30% is lost after implantation but before recognition of a pregnancy by the woman and 10% is lost after clinical confirmation of a pregnancy. Eventually only 30% of all conceptions will result in a live born child (Macklon *et al.*, 2002). Within one year, around 90% of the couples trying to achieve a pregnancy, will succeed (Taylor, 2003). However, about 10% of the couples is confronted with subfertility, i.e. not achieving a pregnancy in spite of regular intercourse aimed at conception for more than 12 months (Beurskens *et al.*, 1995; Evers, 2002).

The aetiology is diverse: ovulation failure (25%), semen anomalies (30%), tubal factors (20%), endometriosis (5%) and unexplained (25%). This exceeds 100% as some couples have more than one cause of subfertility. Roughly, it can be said that in 30% of the couples subfertility is caused by a female factor, in 30% by a male factor and in another 30% by a combination of both a male and a female factor. For the remaining 10% the cause is unknown (Cahill and Wardle, 2002).

Depending on the diagnosis, an appropriate treatment like ovulation induction, intrauterine insemination (IUI) or in vitro fertilization (IVF) with or without intracytoplasmic sperm injection (ICSI) can be applied. The latter is the last resort for severe subfertility cases and longstanding unexplained subfertility. More than 257,000 IVF cycles are performed each year in Europe, of which almost 15,000 in The Netherlands. Nowadays, depending on the country, 1.3-4.2% of the newborns in Europe are born after the application of assisted reproductive technologies (ART) (Nyboe Andersen *et al.*, 2006).

Risks and complications of IVF and ICSI

Although effort is made with IVF and ICSI to mimic the natural situation as closely as possible, there are stages that are different in comparison to the natural cycle. These differences entail a number of potential risks for the patient as well as for the embryo and/or the children to be born.

Ovarian stimulation: The administration of gonadotrophins for follicle development results in approximately 0.2-1% of the patients in severe ovarian hyperstimulation syndrome (OHSS). This can, due to the accompanying electrolyte disorders and thrombo-embolic accidents, even lead to death of the patient (Abramov *et al.*, 1999; Delvigne and Rozenberg, 2003).

Fertilization and in vitro culture: The invasive ICSI technique and the accompanying injection of biochemical components together with the spermatozoon, might be the cause of the increase in aneuploidy and chromosomal anomalies reported in ICSI children (Liebaers *et al.*, 1995; Loft *et al.*, 1999; Bonduelle *et al.*, 2002). It is however more likely that they are caused by the higher aneuploidy rate in the sperm cells of subfertile men (Bonduelle *et al.*, 2002).
Also the embryo culture is reason for concern. Components of the culture medium can disrupt the imprinted expression of genes in mice (Khosla *et al.*, 2001). The prevalence of imprinting disorders in human is 2.9% - 4.2% after ART (Gosden *et al.*, 2003; Sutcliffe *et al.*, 2006), although the study population in both studies is very small (n=214 and 213 respectively).

Multiple embryo transfer: To increase the success rate for IVF, ovarian stimulation was introduced to generate multiple embryos (Speirs *et al.*, 1983; Gronow *et al.*, 1985). The transfer of multiple embryos resulted in an increase in the multiple pregnancy rate (multiple PR), as described in paragraph 1.3. In Europe in 2002, an average of 2.2 embryos was transferred per cycle, resulting in 75.5% singleton, 23.2% twin and 1.3% triplet pregnancies (Nyboe Andersen *et al.*, 2006). In the USA, an average of 2.8 embryos was transferred in 2002, resulting in 64.8% singleton, 31.7% twin and 3.5% triplet or higher order pregnancies (Wright *et al.*, 2005). This means that around 40% of the children born after ART in Europe and 55% in the USA originated from a multiple pregnancy, compared to 2-3% in the general population (Bergh *et al.*, 1999). Regarding the incidence and the associated complications as described in paragraph 1.4, a multiple pregnancy is regarded as one of the most serious complications of IVF treatment. This thesis is focussed on the reduction of the multiple and especially the twin PR.

Aetiology and prevalence of twin pregnancies

Twin gestations can be divided into monozygotic and dizygotic, depending on the number of oocytes that have been fertilized. An embryo resulting from one fertilized oocyte can split, resulting in a monozygotic (MZ) twin, whereas the fertilization of two oocytes results in a dizygotic (DZ) twin (ESHRE Capri Workshop, 2000).

As *MZ twinning* is a random embryological event, largely independent of environmental factors, the rate remained constant throughout the world at 4 per 1000 live births (ESHRE Campus Course, 2001). In ART live births this rate is doubled (Schachter *et al.*, 2001). MZ twinning is positively correlated with ovarian stimulation (Derom *et al.*, 1991; Schachter *et al.*, 2001),

blastocyst transfer (Milki *et al.*, 2003; Jain *et al.*, 2004) and zona pellucida micromanipulation (Slotnick and Ortega, 1996; Abusheikha *et al.*, 2000).

Spontaneous *DZ twinning* (with an incidence of 1.2% (ESHRE Capri Workshop, 2000)) is affected by more factors like maternal age, parity, ethnic background, nutrition and heredity (Bortolus *et al.*, 1999). Its incidence increases four-fold from the maternal age of 15 to 37 years and two-fold from parities of 0 to 10 (Tong and Short, 1998). Also ethnicity is involved as the highest rates are reported in Nigeria (50/1000 live births) (ESHRE Capri Workshop, 2000) and the lowest in Japan (4/1000 live births) (Wenstrom and Gall, 1988). In general, the DZ twin rate is about 8/1000 in whites, about twice as large in the black community and less than half as large in Asians (Bortolus *et al.*, 1999). Explanations for this ethnic difference might be differences in basal FSH concentration (Nylander, 1981), genetic predisposition or malnutrition (Bortolus *et al.*, 1999).

Before the onset of ART, the multiple PR was gradually declining in several western countries (Botting *et al.*, 1987; Tuppin *et al.*, 1993; Luke, 1994). This reduction was attributable to a decrease in the incidence of risk factors like enhanced maternal age and multiparity (Senat *et al.*, 1998). However, in the mid-1970s the multiple PR started to increase again. This was largely due to the introduction of subfertility treatment (Tuppin *et al.*, 1993; Dunn and Macfarlane, 1996). The first series of IVF treatments, performed in the late seventies, was attempted using the single oocyte from the natural cycle. The PR was very low (4/32=13%), as only three children were delivered of which one died after a premature birth (Edwards, 1996). Soon it was realised that the success rate per cycle could be increased by transferring multiple embryos, instead of one (Speirs *et al.*, 1983; Gronow *et al.*, 1985). To achieve these multiple embryos, ovarian stimulation was introduced as a part of the IVF treatment (Edwards, 1996). Fifteen to twenty years after this introduction the twin PR was increased 1.3-1.7 fold and the higher order multiple PR 2-4 fold in several European countries and the USA (Van Duivenboden *et al.*, 1991; Tuppin *et al.*, 1993; Dunn and Macfarlane, 1996; Westergaard *et al.*, 1997; Bergh *et al.*, 1999; Martin and Park, 1999). These increases were not caused by multiple embryo transfer in IVF alone. Also other types of subfertility treatment, like ovulation induction in anovulatory women or superovulation in combination with intrauterine insemination (IUI) were involved as well as an increase in maternal age at childbirth. Bergh *et al.* (1999) demonstrated that the advanced age of childbearing women was responsible for one third of the increase in twin pregnancies. Another third is due to an increase in the use of IVF and the remaining third because of an increase in ovarian stimulation. This is in accordance with the findings of a study of 169 multiple pregnancies in UK, showing that 34% resulted from ovarian stimulation without IVF and 35% with IVF (Doyle, 1996).

Obstetric risks, neonatal complications and costs of twin pregnancies

Maternal complications

Table I summarizes data regarding the incidence and risk of maternal complications in singleton and twin pregnancies. Besides a small but significantly increased risk of *maternal mortality* (Senat *et al.*, 1998), women carrying a twin are at an increased risk of several disorders and complications like hypertension, hemorrhage, anemia, and delivery by Caesarean section (Spellacy *et al.*, 1990; Stones *et al.*, 1993; Santema *et al.*, 1995; Senat *et al.*, 1998; Sibai *et al.*, 2000; Sebire *et al.*, 2001). *Hypertension* is the major maternal complication in twin pregnancies (Santema *et al.*, 1995; Senat *et al.*, 1998). A distinction has to be made between non-proteinuric and proteinuric hypertension (pre-eclampsia) (Senat *et al.*, 1998; Norwitz *et al.*, 2005). The latter is accompanied by higher maternal and fetal morbidity and mortality as compared to the first (Senat *et al.*, 1998; Norwitz *et al.*, 2005). Twin pregnancies also have an increased risk of *antepartum hemorrhage* (partly caused by abruptio placentae) (Spellacy *et al.*, 1990) and *postpartum hemorrhage* (Stones *et al.*, 1993; Sebire *et al.*, 2001). This is also visualised by the increased incidence of transfusions (8% for twin and 2.5% for singleton deliveries) (Kinzler *et al.*, 2000) and *anemia* (9.4% vs 4.1%, respectively) (Spellacy *et al.*, 1990). Finally an almost three-fold risk of *Caesarean section*, which has an increased risk of maternal morbidity and mortality, has been reported (Sebire *et al.*, 2001).

Neonatal outcome

The most frequently occurring complications in twin pregnancies are *preterm delivery* and low *birthweight* (Table II). The mean birthweight of a twin is 2300g against 3300g for a singleton (Spellacy *et al.*, 1990; Doyle, 1996; Kinzler *et al.*, 2000). The mean gestational age of a twin pregnancy is 35.6 weeks, while a singleton pregnancy is delivered on average at 39.0 weeks (Kinzler *et al.*, 2000). The aetiology of preterm delivery in twin births, in a study analyzing 432 twin pregnancies, was spontaneous (54%), premature rupture of membranes (22%) and induced deliveries due to maternal hypertension, fetal growth restriction or fetal death (23%) (Gardner *et al.*, 1995). Preterm delivery and low birthweight are the major causes of neonatal morbidity and mortality.

Both the early fetal death rate and the still birth and neonatal *mortality* rate are increased in twin pregnancies (Table II). Early fetal demise presenting as vanishing sacs is identified especially after ART pregnancies, as these pregnancies are followed-up more frequently during early pregnancy as compared to spontaneous pregnancies. Of the pregnancies with two fetal sacs, approximately 30% will result in one fetal sac and < 10% in no fetal sac (Landy and Keith, 1998). For spontaneous pregnancies, De Neubourg *et al.* (2004) reported a first trimester pregnancy loss of 15.4% after IVF.

Besides the increased mortality rate, also the increased morbidity is attributable to the low birth weight and preterm delivery. Among other complications, a 6.6-fold risk of *respiratory distress syndrome* and a 4.5-fold increase in *intraventricular hemorrhage* was observed in twins compared to singletons (Gardner *et al.*, 1995). This increase in neonatal complications results in an increased admittance to neonatal intensive care units (NICU) of twin children (20.6% of twin and 3.9% singleton) (Sebire *et al.*, 2001).
Twins also have an increase in *long-term medical and developmental problems* like chronic illness, communicative disorders and neurological impairments (Luke and Keith, 1992), with *cerebral palsy* (CP) being the most important (Petterson *et al.*, 1993). CP is characterized by movement disabilities attributed to disturbances in the developing fetal or neonatal brain. However, Pinborg *et al.* (2003) did not found a difference between IVF/ICSI twin or singletons regarding severe neurological disabilities. Due to shared parental attention the language development can be adversely affected (Mittler, 1976; Pinborg *et al.*, 2003) and the death of one of the twins can lay a psychological burden on the other twin (Bryan and Read, 1995). Furthermore, twins are a predictor for more marital stress (more crises in the relationship and thoughts about divorce) as well as for less marital benefit (Pinborg *et al.*, 2003).

Economic implications
Due to these maternal and neonatal complications, the healthcare costs are high in multiple pregnancies. It has been determined that these costs up to six weeks after delivery are €13,469 per twin compared to €2,549 per singleton pregnancy (Lukassen *et al.*, 2004). This difference is mainly caused by the maternal hospitalization, resulting from the increased incidence of preterm delivery, and the associated admittance of the neonates to the NICU together with diagnostic procedures and drug therapies. For a minor part, the difference could be attributed to extra examinations during the antenatal period. The difference in costs will increase even more when the lifetime costs for handicaps, long-term chronic complications and developmental disabilities are included.

For instance for the hospital care utilization of IVF/ICSI twins vs singletons an odds ratio (OR) of 2.44 was determined. When the premature infants were excluded, this OR was still 1.4 (Pinborg *et al.*, 2004). Wolner-Hanssen calculated that the average costs for care of severely handicapped children was almost 14 times higher for twin pregnancies (€20477) compared to singleton pregnancies (€1489) (Wolner-Hanssen and Rydhstroem, 1998; Lukassen *et al.*, 2005).

Table I: Maternal complications in singleton versus twin pregnancies

Complication	Cohort size		Incidence		RR (TP/SP)	Reference
	SP	TP	SP	TP		
Maternal mortality	NA[1]	NA[1]	5.2/100,000	14.9/100,000	2.8	(Senat *et al.*, 1998)
Hypertension	2946	684	6.3%	12.9%	2.0	(Sibai *et al.*, 2000)
	5119	1253	5.6%	12.9%	2.3	(Spellacy *et al.*, 1990)
	187	187	13%	21%	1.6	(Santema *et al.*, 1995)
Pre-eclampsia	2946	684	4.9%	12.7%	2.6	(Sibai *et al.*, 2000)
	417,542	5416	0.8%	2.9%	3.6	(Sebire *et al.*, 2001)
	187	187	6.5%[a]	6%[a]	0.9	(Santema *et al.*, 1995)
Abruptio placenta	5119	1253	0.8%[a]	2.2%[a]	2.8	(Spellacy *et al.*, 1990)
Postpartum hemorrhage (>1000ml)	36,630	817	1.2%	5.5%	4.6	(Stones *et al.*, 1993)
	417,542	5416	1.6%	6.6%	4.1	(Sebire *et al.*, 2001)
Anemia	5119	1253	4.1%	9.4%	2.3	(Spellacy *et al.*, 1990)
Caesarean section	417,542	5416	9.3%	26.8%	2.9	(Sebire *et al.*, 2001)

SP: Singleton pregnancy, TP: twin pregnancy, RR: relative risk, NA: Not available
[1] 1995 Concerted European Investigation (MOMS)
[a] superscripts within one row are not significantly different ($P > 0.05$)

Table II: Neonatal complications in singleton versus twin pregnancies

Complication		Cohort size		Incidence[c]		RR (TP/SP)	Reference
		SP	TP	SP	TP		
Preterm delivery	<37 weeks	417,542	5416	6.2%	45.2%	7.3	(Sebire *et al.*, 2001)
		33,873	432	9.6%	54%	5.6	(Gardner *et al.*, 1995)
	<32 weeks	417,542	5416	1.1%	8.5%	7.7	(Sebire *et al.*, 2001)
Birthweight < 2500g		5119	1253	7.9%	51.0%	6.5	(Spellacy *et al.*, 1990)
		NA[a]	NA[a]	5.9%	53.1%	9.0	(Kinzler *et al.*, 2000)
		417,542	5416	6.0%	52.3%	8.7	(Sebire *et al.*, 2001)
Mortality	stillbirth	NA	NA	4.4‰	14.2‰	3.2	(Doyle, 1996)
	neonatal (0-27 days)	NA	NA	3.7‰	26.7‰	7.2	(Doyle, 1996)
Respiratory distress syndrome		33,873	432	2.4%	15.8%	6.6	(Gardner *et al.*, 1995)
Intraventricular hemorrhage		33,873	432	0.2%	0.9%	4.5	(Gardner *et al.*, 1995)
Neonatal Intensive Care Unit		11,986	1135	15%	48%	3.2	(Callahan *et al.*, 1994)
Handicaps	severe	NA[b]	NA[b]	19.7‰	34.0‰	1.7	(Luke and Keith, 1992)
	moderate	NA[b]	NA[b]	70.6‰	92.3‰	1.3	(Luke and Keith, 1992)
Cerebral Palsy		228,329	5288	1.6‰	13.2‰	8.3	(Petterson *et al.*, 1993)

SP: Singleton pregnancy, TP: twin pregnancy, RR: relative risk, NA: Not Available
[a] 1995-1996 United States data
[b] 1988 U.S. birth cohort
[c] All incidences within one row are significantly different ($P < 0.05$)

The approach to prevention of twin pregnancies - Single embryo transfer

In the nineties, when the iatrogenic increase in multiple pregnancies due to multiple embryo transfers was recognized as a complication of IVF, several observational studies focussed on the reduction of the number of embryos transferred. They all found that when reducing the number of transferred embryos from three to two, the multiple PR was reduced without diminishing the overall ongoing PR (Waterstone *et al.*, 1991; Staessen *et al.*, 1993; Fujii *et al.*, 1998; Templeton and Morris, 1998; Dean *et al.*, 2000). The reduction in multiple PR was predominantly due to a virtual elimination of the triplet pregnancies. The rate of twin pregnancies remained constant between 20 and 30% (Staessen *et al.*, 1993; Fujii *et al.*, 1998; Dean *et al.*, 2000; Nyboe Andersen *et al.*, 2005).

The next step to further reduce the multiple PR (i.e. twin PR) was the transfer of a single embryo (Single Embryo Transfer, SET). In the beginning, SET was applied when there was only one embryo available (compulsory SET, cSET), leading to low PRs of 10.3% (Giorgetti *et al.*, 1995). But as the overall PR after IVF increased due to the improvement of culture media, a better identification of viable embryos and an improved transfer procedure (Gerris *et al.*, 1999), SET was also introduced in patients who had more than one embryo available (i.e. elective SET, eSET) (Gerris *et al.*, 1999; Vilska *et al.*, 1999).

Gerris and colleagues (1999) were the first to conduct a randomized controlled trial (RCT) between eSET and double embro transfer (DET). Only a selected group of good-prognosis patients, defined by age and number of good morphology embryos was included in the study (Table III). After this study more clinics, especially in Belgium and Finland started with the application of eSET in a selected population of twin prone patiens (Table III and IV) (Gerris *et al.*, 1999; Martikainen *et al.*, 2001). The RCTs performed before the start of our study described in chapter 3 (i.e. before 2002), showed that the transfer of only one good quality embryo resulted in a lower PR as compared with the transfer of two good quality embryos, although the difference was not significant in both studies (Table III). As the PRs after eSET were still acceptable and the twin pregnancies were prevented, it was concluded that eSET should be the transfer policy of choice in this subgroup of good prognosis patients (Gerris *et al.*, 1999; Martikainen *et al.*, 2001)

Vilska *et al.* (1999) were the first to publish on the influence of applying an eSET policy on the overall outcome in an IVF programme. eSET was performed for medical reasons, risk of OHSS and patient's wish (Table IV). Also Tiitinen *et al.* (2001) applied eSET only for patient's wish and medical reasons (diabetes mellitus, uterine malformation and indication for prenatal diagnosis).

The PRs between the eSET and DET group were comparable in both studies, with acceptable overall PRs and slightly reduced twin PRs (Table IV) (Vilska *et al.*, 1999; Tiitinen *et al.*, 2001).
Gerris et al. (2002) showed the results from the preceeding four years, in which the proportion of eSET was gradually increased in their clinic. It started with the RCT in women < 34 years of age in their first IVF or ICSI cycle with at least two top quality embryos (Gerris et al., 1999). After this study a new study was started, in which the criteria for eSET were liberated. Women < 38 years of age in their first IVF or ICSI cycle were allowed to choose whether they wanted one or two embryos transferred in case a top quality embryo was available. A small subgroup consisted of patients who preferred eSET, irrespective of the availability of a top quality embryo. Due to this judicious implementation of eSET, the overall PR remained similar over the years, while the twin PR was reduced from 29.5% in 1998 to 16.3% in 2001 (Gerris et al., 2002). Also de Sutter et al. (2003) reported a reduction in twin PR (from 30% to 21%) after a gradual implementation of eSET over a four-year period, while the PR (35%) remained similar.

Despite all efforts in implementing eSET, the twin PR did not fall below 15%. Although this is a great step forward as compared with the transfer of two embryos (25-30%), this rate is, in our opinion, unacceptable regarding the risks and complications that are involved in a twin pregnancy. This was also confirmed in the following recommendation made at the ESHRE campus course on the prevention of twin pregnancies: "A twin (and higher order multiple) PR of 25% or more is not acceptable and must induce practitioners to elaborate an individualized embryo transfer strategy, aiming at reducing this incidence to perhaps around 10%" (ESHRE Campus Course, 2001).

The approach to prevention of twin pregnancies – Patient and embryo selection for eSET

Patient selection
Several studies have focussed on defining predictors for patients at an increased risk for a twin pregnancy and therefore candidates for eSET. Both Strandell *et al.* (2000) and Hunault *et al.* (2002) found after a multivariate analysis that female age and number of good quality embryos transferred were the most important factors, independently predictive of birth and multiple birth. Number of previous IVF attempts and duration of infertility were negatively associated with the chance of a birth or a multiple birth (Templeton and Morris, 1998). This was confirmed by Staessen and collegues (1993), who analysed PRs between DET and triple embryo transfer and found no difference in patients < 37 years old in their first IVF attempt with at least three good quality embryos available for transfer (Staessen *et al.*, 1993). Also Coetsier and Dhont (1998) found that age <

36 years, first, second or third IVF or ICSI cycle, more than three embryos available for transfer and the quality of the transferred embryos (i.e. at least 4 on a scale of 1-5), are associated with good prognosis.

Embryo selection based on morphologic parameters
To define the morphological characteristics of a good quality embryo, 23 DETs resulting in a twin pregnancy were analysed. The resulting characteristics are 4 or 5 blastomeres on day 2, seven or more on day 3 and ≤ 20% fragmentation (Van Royen *et al.*, 1999). Several other studies focussed on the pregnancy predictive value of morphological characteristics of the zygote and embryo at different stages of development. The morphological parameters for the zygote include the number of nuclear precursor bodies (NPB) and their distribution in the pronuclei (Scott and Smith, 1998; Tesarik *et al.*, 2000; Wittemer *et al.*, 2000). Three or more NPBs and an equal distribution in both pronuclei are positively correlated with implantation and blastocyst development. There was only one study analysing SETs, but in this study no significant differences in implantation rate between the zygote scoring classes were found (Salumets *et al.*, 2001). Another parameter analysed, is the time of the first cleavage division of the zygote and the development rate during the forthcomings days. Whether or not the first cleavage division could be seen within 25-27h after insemination or ICSI influenced the pregnancy and implantation rate (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Bos-Mikich *et al.*, 2001; Lundin *et al.*, 2001; Sakkas *et al.*, 2001; Fenwick *et al.*, 2002; Tsai *et al.*, 2002). Early cleavage (EC) embryos resulted more often in a pregnancy than non-early cleavage (NEC) embryos. However, most studies analysed multiple embryo transfers of which many cycles had transfers with both an early and a non-early cleavage embryo. Therefore it remains uncertain whether the pregnancy or implantation can be attributed to the early cleavage embryo. At the second day of development, an embryo consisting of four cells with little or no fragmentation has the highest chance to implant (Ziebe *et al.*, 1997; Van Royen *et al.*, 1999). Furthermore, when there are no multinucleated blastomeres (MNBs) (Pelinck *et al.*, 1998; Van Royen *et al.*, 2003) and when the blastomeres are even sized, the implantation potential of the embryo increases even more. Unevenly sized blastomeres might point towards aneuploidy (Hardarson *et al.*, 2001). In clinics transferring at day 3, the most optimal cell number is 7-8, also with little or no fragmentation and no multinucleated, even sized blastomeres (Van Royen *et al.*, 1999).

Embryo selection based on non-morphologic parameters
Only the oberservation of embryo morphology on the few days before transfer cannot fully reflect the implantation potential of the embryo. This for instance emerges as around 30-40% of the morphologically normal embryos and blastocysts display aneuploidy, which reduces the implantation chance (Munne *et al.*, 1995; Magli *et al.*, 2000).

Table III: Selection criteria and pregnancy outcome in RCTs, published before the onset of our studies, on one cycle eSET versus one cycle DET (fresh embryos)

	Selection criteria for RCT				eSET		DET	
	age	No of good quality embryos	Cycle	*n*	PR (%)	Twin rate (%)	PR (%)	Twin rate (%)
Gerris *et al.*, 1999	< 34	≥ 2	1	53	10/26 (38.5)[a]	1/10 (10)	20/27 (74.1)[a]	6/20 (30)
Martikainen *et al.*, 2001[#]	-	≥ 4	1 or 2	101	22/74 (29.7)	1/22 (4.5)	28/70 (40)	11/28 (39.2)
	< 36	≥ 4	1	43				

PR = pregnancy rate
[#] multicenter trial with some centres having aberrant criteria
[a] PRs within one row are significantly different ($P < 0.05$)

Table IV: Selection criteria for eSET and pregnancy outcome in observational studies on eSET and DET, published before the onset of our studies (fresh embryos)

	Selection criteria for eSET		eSET		DET		Overall	
		n	PR (%)	Twin rate (%)	PR (%)	Twin rate (%)	PR (%)	Twin rate (%)
Vilska *et al.* 1999	Patient's wish or medical reasons	816	22/74 (29.7)	0/22 (0)	218/742 (29.4)	52/218 (23.9)	240/816 (29.4)	52/240 (21.6)
Tiitinen *et al.* 2001	Patient's wish or medical reasons	644	49/127 (38.6)	1/49 (2)	203/517 (40.0)	42/203 (20.7)	252/644 (39.1)	43/252 (17.1)

PR = pregnancy rate

To improve their implantation rate, a new selection method was developed: preimplantation genetic diagnosis on chromosomal aneuploidies (PGD-AS). Although a retrospective study in these patients showed a positive effect of PGD-AS on the PRs (Munne *et al.*, 2003), in a large randomized controlled trial no positive effect could be found (Staessen *et al.*, 2004). To further achieve some characteristics of the embyo not visible from the outside, laboratories focus on biochemical markers in culture medium and follicular fluid. To assess the metabolic activity of an embryo, the uptake of nutrients from the surrounding medium or the secretion of metabolites can be examined. Gardner *et al.* (2001) determined that embryos developing into a blastocyst consumed more glucose and pyruvate and that the quality of the blastocyst was positively related to the glycose uptake. Furthermore, Van den Bergh *et al.* (2001) showed that blastocysts resulting in a pregnancy had a higher glucose uptake compared to blastocysts not resulting in a pregnancy.

There is increasing evidence that the heterogeneity of the follicular micro-environment has a significant impact on oocyte competence and embryonic development. Follicular fluid obtained after oocyte retrieval can be analysed for several factors. It appeared that the concentration of hormones (17β-estradiol, LH, growth hormone, prolactin), growth factors (insulin-like growth factor-I) and cytokines (interleukin-1) in follicular fluid are correlated with pregnancy (Mendoza *et al.*, 2002). Also ATP content of the oocyte and dissolved oxygen content of the follicle fluid have been suggested as parameters for oocyte/embryo development (Van Blerkom *et al.*, 1995; Van Blerkom *et al.*, 1997).

Besides the biochemical markers of the intrafollicular environment, also vascularization of the follicles has been examined as a potential parameter for embryo quality. The vascularization of pre-ovulatory follicles is related to oocyte oxygenation (Van Blerkom *et al.*, 1997) and can differ between the follicles of one patient. Nargund *et al.* (1996) found by Doppler imaging of the follicular blood flow that oocytes from poorly vascularized follicles developed in morphologically inferior embryos as compared to well vascularized follicles. Several other studies confirmed a positive relationship between perifollicular vascularization and pregnancy (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997; Borini *et al.*, 2004). Pregnancies were only achieved with embryos from follicles where vascularity was detected in > 50% of the follicular circumference and live births only from oocytes with > 76% follicular vascularity (Chui *et al.*, 1997). In addition, both Chui *et al.* (1997) and Van Blerkom *et al.* (1997) reported a significant higher incidence of aneuploidy and spindle defects in oocytes derived from follicles with poor vascularization as compared to well-vascularized follicles.

Aim and outline of thesis

As a twin pregnancy is one of the most adverse outcomes of an IVF treatment, affecting around 25% of the patients, the studies described in this thesis focussed on the reduction of the twin PR after IVF. The problem of and possible solutions for the prevention of twin pregnancies will be viewed from different angles. The general aim of the thesis was to analyse several transfer strategies with eSET from a clinical and a cost-effectiveness point of view and to improve the selection of the single best embryo for eSET.

In a first attempt to reduce the twin PR, the influence of eSET applied in all cycles, instead of only the first one, in a selected group of good prognosis patients was analyzed (chapter 2). To further reduce the twin PR without gradually liberating the selection criteria, eSET was applied in the first cycle of an unselected group of patients. The clinical outcome as well as the cost-effectiveness of this strategy was analysed by performing an RCT of eSET versus DET (chapter 3 and 4). Finally, another strategy to reduce the twin PR, the application of eSET in the first cycle of all patients and eSET or DET depending on patient and embryo characteristics in the following cycles, was analysed (chapter 5).

To optimize PRs after IVF, and especially after eSET, the embryo with the highest implantation potential has to be selected. This selection was improved by focussing on early cleavage (chapter 6). Since also intra-follicular factors influence embryo development, the final objective of this thesis was to study whether differences in follicular gene expression reflect embryo viability (chapter 7).

Elective single embryo transfer (eSET) policy in the first three IVF/ ICSI treatment cycles

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Abstract

BACKGROUND: Elective single embryo transfer (eSET), applied in the first or second IVF cycle in young patients with good quality embryos, has been demonstrated to lower the twin pregnancy rate (PR), while the overall PR is not compromised. It is as yet unclear whether eSET could be the preferred transfer policy in all treatment cycles, or that it should be restricted to the first or first two cycles.

METHODS: eSET policy (when two or more embryos were available, at least one of them being of good quality) was offered to patients younger than 38 years in the first three treatment cycles. Retrospectively, treatment cycle outcome was studied.

RESULTS: In 326 patients, 586 treatment cycles were performed (326 first, 168 second and 92 third treatment cycles). In 65 cycles (11%), eSET could not be applied because there was either no fertilization, or only one embryo available. In the remaining 521 cycles, eSET was performed in 111 cycles (19%), while in 410 cycles, no good quality embryo was available resulting in the transfer of two embryos (double embryo transfer, DET). No significant differences in ongoing PRs after transfer of fresh embryos were observed between eSET and DET in the first (both 33%), second (36 and 23%, respectively) and third treatment cycles (20 and 24%, respectively). In significantly more eSET cycles compared to DET cycles, embryos could be frozen. This resulted in a significantly higher cumulative PR after eSET compared to DET.

CONCLUSION: In patients younger than 38 years with at least one top quality embryo, eSET can be the transfer policy of choice in at least the first three treatment cycles, since the PRs obtained in each treatment cycle are comparable to those after DET.

Introduction

Multiple pregnancy rates (PRs) are high in assisted reproductive technology (ART) cycles. Recently, the European IVF-monitoring programme of the European Society of Human Reproduction and Embryology (ESHRE) reported a 26.3% multiple delivery rate in 258,460 IVF/ICSI treatment cycles initiated during 1999 in 22 countries (ESHRE, 2002). The majority of multiple deliveries after IVF and ICSI concern twins, representing 24.0% of all deliveries (ESHRE, 2002). Twin pregnancies must be regarded as a serious complication of ART cycles, with relatively high risks of health problems in the children (both during the pre- and perinatal period and later in life) and their mothers, social problems, and high economic costs (ESHRE Capri Workshop, 2000; Gerris *et al.*, 2004).
The high twin PR after IVF is the result of the current standard practice of transferring more than one embryo. The elective transfer of only a single embryo (eSET) has been shown to be an effective method to reduce the incidence of twin pregnancies in twin-prone IVF/ICSI patients without compromising the overall ongoing PR (Vilska *et al.*, 1999; ESHRE Campus Course, 2001; Gerris *et al.*, 2001; Martikainen *et al.*, 2001; Tiitinen *et al.*, 2001; Gerris *et al.*, 2002; De Neubourg and Gerris, 2003; Tiitinen *et al.*, 2003).
It was recently recommended by the ESHRE consensus meeting on risks and complications in ART that eSET should be proposed in the first and second treatment cycles (Land and Evers, 2003). Since 2003 in Belgium, SET in the first, and eSET in the second treatment cycle is mandatory in patients younger than 36 years of age, to obtain maximal reimbursement of the costs of IVF treatment (Ombelet, 2004). However, little information is available on eSET results to substantiate this proposal to limit eSET to the first two treatment cycles. In the present study we evaluate the results per treatment cycle number in a cohort of patients in whom eSET was offered as the standard transfer policy during the first three treatment cycles.

Materials and methods

Patients
All IVF/ICSI patients who started their first treatment cycle in the period from July 2000 until December 2001 in the academic hospital Maastricht were included in this study. A treatment cycle was defined as an ovarian stimulation cycle which resulted in an ovum pick-up. In the study period eSET was offered as our standard transfer policy (see below) to patients younger than 38 years at the time of transfer, irrespective of the rank of the cycle and of previous IVF results. Only patients who were 38 years or older, patients with a strong wish for the transfer of either one or two embryos, patients who had a medical or socio/psychological reason to prevent

a twin pregnancy, and patients requesting preimplantation genetic diagnosis (PGD) were excluded from the standard eSET policy.
In the study period, the costs of the first three IVF/ICSI treatment cycles were fully reimbursed in The Netherlands. For this reason the study has been limited to the first three treatment cycles, including the transfer of frozen embryos, if any.

Ovarian stimulation protocol
Patients were down-regulated with daily s.c. injections of triptorelin (Decapeptyl; Ferring B.V., Hoofddorp, The Netherlands) or nafarelin intranasally (Synarel; Searle BV, Maarssen, The Netherlands) according to the long protocol. To stimulate multiple follicular development, recombinant FSH (Puregon, Organon, Oss, The Netherlands) was used. Follicle growth was monitored by ultrasound and 5000 IU of hCG (Pregnyl, Organon, Oss, The Netherlands) was given as soon as at least three follicles were $\geq$18 mm. Ultrasound-guided oocyte retrieval was performed 36 h after hCG administration. The luteal phase was supported by progesterone (Progestan, Organon, Oss, The Netherlands) 600 mg daily intravaginally, starting at the day of ovum pick-up and continued for 14-16 days. In the case of pregnancy 600 mg Progestan was continued for three more weeks.

Laboratory and embryo transfer procedures
IVF and ICSI procedures used have been described in detail earlier (Dumoulin *et al.*, 2001). Oocytes and embryos were cultured individually in 5 µl droplets covered by mineral oil in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2 in sequential culture media either from Vitrolife (Göteborg, Sweden) or from Cook (Eight Mile Plains, Queensland, Australia). Embryo transfer was routinely performed on day two or day three after ovum retrieval. Embryos were evaluated at 23-26h post-injection (in case of ICSI) or 25-28h post-insemination (in case of IVF), 41-45h post-injection/insemination and in case of day three transfer also at 65-69h post-injection/insemination. For each embryo originating from a normally fertilized oocyte, an embryo score was calculated on the basis of morphological grade (1 to 4, with grade 4 being the best grade, using the grading system of Bolton *et al.* (1989)), number of blastomeres and presence or absence of multinucleated blastomeres (MNBs). Embryos that had reached the 4- or 5-cell stage on day 2 or the 8-cell stage on day 3, in combination with having the best morphological grade (regular, even sized blastomeres with < 20% fragmentation) and an absence of MNBs were classified as good quality embryos (Van Royen *et al.*, 1999). When patients were eligible for standard eSET policy and if at least one good quality embryo was available, only a single embryo was transferred (eSET). In all other cases, two embryos, if available, were transferred (double embryo transfer, DET). Cryopreservation of supernumerary embryos was performed on the

third day after ovum retrieval. Only embryos which had reached the 8-cell stage and which were considered to be of sufficient morphological quality (grades 3 or 4) were cryopreserved. After thawing, two embryos, if available, were transferred, also in cases in which in the fresh transfer eSET was applied.

Pregnancy
An hCG pregnancy test with a detection limit of 50 IU/l in urine was performed 14-16 days after embryo transfer and patients with a positive test had an ultrasound examination 3 weeks later. Patients were asked to report an abortion occuring after this ultrasound examination immediately. In this study an ongoing pregnancy was defined as the presence of at least one intrauterine gestational sac with fetal heartbeat on ultrasound at ~ 7/8 weeks gestation and no report of pregnancy loss before 12 weeks gestation.

Statistics
The χ^2 test with Bonferroni correction was used to compare the PRs. Differences were considered significant at $P < 0.05$.

Results

In our IVF center, in the period of July 2000 until December 2001, 388 patients underwent their first IVF/ICSI treatment cycle. The results of 62 patients (16%) were not included in the present study for the following reasons: 15 patients applied for PGD, 43 patients had reached the age of 38 years at the time of their first or subsequent IVF treatment cycles and four patients had either a strong wish for the transfer of one or two embryos or had medical or socio/psychological reasons for single embryo transfer.
The results of the first treatment cycles (performed in the period July 2000 until December 2001) and of all eventual subsequent second or third treatment cycles or frozen embryo replacement cycles (performed in the period July 2000 until May 2004) of the 326 patients who accepted our standard eSET policy can be found in Table I. In these patients, after transfer of fresh embryos, 159 ongoing pregnancies were achieved (49%), of which 40 (25%) were twin pregnancies. In 175 patients (54%), an ongoing pregnancy was achieved, either after transfer of fresh embryos in the first, second or third treatment cycle, or after transfer of cryopreserved embryos.
The 326 patients eligible for eSET policy underwent 586 treatment cycles (1.8 cycle per patient). Total fertilization failure was found in 26 cycles (4%), while in 39 cycles (7%), only one embryo was obtained and transferred

(compulsory SET, cSET). In 521 treatment cycles, more than one embryo was available. In 111 of these cycles (21%), at least one good-quality embryo was present and consequently only one embryo was transferred (eSET). In this group, 39 positive pregnancy tests (35%) were achieved, of which four ended in an early abortion (10%). In 410 cycles, two embryos were transferred (DET). In this group 142 positive pregnancy tests (35%) were achieved, of which 23 ended in an early abortion (16%). The PRs after one, two or three cycles were respectively 33, 36 and 20% for eSET and 33, 23 and 24% for DET. The differences between the first, second and third eSET cycle and between the first, second and third DET cycle were not statistically significant. Neither are the differences between eSET and DET in each treatment cycle rank group.

In 112 cycles at least one surplus embryo of good morphology was available after transfer and subsequently frozen. After eSET significantly more often embryos could be cryopreserved as compared to after DET (53% and 13% of the treatment cycles, respectively). In table I, pregnancy results are shown for 65 thaw cycles performed in patients who did not get pregnant in any of their transfers of fresh embryos. This resulted in 16 ongoing pregnancies (25%). In 12 patients, thawing has not yet been performed because of the patients' wishes. The cumulative ongoing PRs after fresh and frozen transfers were significantly different between eSET and cSET and between eSET and DET (13% cSET, 41% eSET and 30% DET). An additional 35 patients who became pregnant from fresh embryos, had embryos cryopreserved. In seven of these patients embryos have been thawed, resulting in two pregnancies.

After the first cycle, 48 patients (30% of the patients not getting pregnant in the first cycle) dropped out because of medical reasons or patient wishes. After the second cycle the drop-out rate was 36% (27 patients). The Dutch health insurance system only reimburses three IVF/ICSI cycles. Therefore the drop-out rate after the third cycle increased to 75%.

Table I: Results of 586 cycles in 326 patients who entered the standard eSET policy program at the university hospital Maastricht

Treatment cycle rank	Embryos available	Fresh embryos transferred	Cycles (% of total)	Ongoing pregnancies after ET of fresh embryos (% of cycles)	Cycles with freezing (% of cycles)	Thaw cycles	Ongoing pregnancies after ET of cryo-preserved embryos	Total of ongoing pregnancies after ET of fresh or cryopreserved embryos (% of cycles)[2]	Ongoing twin pregnancies (% of ongoing pregnancies)
First	0	0 (no fertilisation)	16 (5)	-	-	-	-	-	-
(326)[1]	1	1 (cSET)	20 (6)	4 (20)	-	-	-	4 (20)	0
	>1	1 (eSET)	55 (17)	18 (33)	31 (56)[a]	18	7	25 (45)	0
	>1	2 (DET)	235 (72)	78 (33)	22 (9)[b]	15	2	80 (34)	26 (33)
Second	0	0 (no fertilisation)	8 (5)	-	-	-	-	-	-
(168)[1]	1	1 (cSET)	15 (9)	1 (7)	-	-	-	1 (7)	0
	>1	1 (eSET)	36 (21)	13 (36)	17 (55)[a]	8	1	14 (39)	0
	>1	2 (DET)	109 (65)	25 (23)	17 (16)[b]	10	3	28 (26)	8 (29)
Third	0	0 (no fertilisation)	2 (2)	-	-	-	-	-	-
(92)[1]	1	1 (cSET)	4 (4)	0	-	-	-	0	0
	>1	1 (eSET)	20 (22)	4 (20)	11 (55)[a]	7	3	7 (35)	0
	>1	2 (DET)	66 (72)	16 (24)	14 (21)[b]	7	0	16 (24)	6 (38)
Totals	0	0 (no fertilisation)	26 (4)	-	-	-	-	-	-
(586)[1]	1	1 (cSET)	39 (7)	5 (13)	-	-	-	5 (13)[c]	0
	>1	1 (eSET)	111 (19)	35 (32)	59 (53)[a]	33	11	46 (41)[d]	0
	>1	2 (DET)	410 (70)	119 (29)	53 (13)[b]	32	5	124 (30)[e]	40 (33)
Totals			586	159 (27)	112 (19)	65	16	175 (30)	40 (23)

[1]The total number of treatment cycles of the given treatment cycle rank.

[2]Proportions with different superscripts within a column are significantly different (χ^2-test, Bonferroni correction, $p < 0.05$).

Discussion

After publication of the encouraging results from the first eSET studies (Gerris *et al.*, 1999; Vilska *et al.*, 1999) showing that acceptable PRs can be obtained when eSET is carried out in a subset of good prognosis patients, and after a brief pilot study in our own clinic, we felt confident in introducing eSET in the year 2000 as our standard clinical ET policy. eSET was offered to all patients younger than 38 years of age, who had at least one good quality embryo, irrespective whether it was their first, second or third treatment cycle.

The PRs obtained in the first two treatment cycles in our study are comparable to those reported by other studies in which eSET was performed in either only the first (Gerris *et al.*, 1999; Vilska *et al.*, 1999) or in the first two treatment cycles (Martikainen *et al.*, 2001; De Neubourg and Gerris, 2003).

It is concluded in several publications that eSET should be limited to the first two cycles (Martikainen *et al.*, 2001; De Neubourg and Gerris, 2003; Land and Evers, 2003). However, our results show that PRs of eSET compared to DET are not significantly different up to and including the third treatment cycle, although it must be noted that the group of patients that received a third treatment cycle is relatively small (four pregnancies in 20 patients receiving only a single embryo). Yet, the PRs in the third treatment cycle in both the eSET and the DET groups are decreased compared with the previous treatment cycles. These decreasing PRs in successive IVF cycles were already reported by others (Tan *et al.*, 1992; Templeton *et al.*, 1996). Our results confirm earlier publications showing that cryopreservation clearly improves the cumulative PR after eSET. (Martikainen *et al.*, 2001; Tiitinen *et al.*, 2001). In our study, in significantly more eSET cycles could embryos be cryopreserved compared to DET cycles (53 and 13% respectively). After thawing, two embryos, if available, were transferred. The reason for this was that cryopreserved embryos have a lower potential for implantation and therefore were not considered to be 'good quality' embryos, which is a necessary condition for eSET (Levran *et al.*, 1990; Edgar *et al.*, 2000a; Edgar *et al.*, 2000b). At present, almost all embryos, from patients who did not became pregnant from fresh embryos, have been thawed. A significantly higher cumulative PR in the eSET group can be seen, caused by the additive effect of frozen embryo transfers.

In order to improve the results after eSET, effort is taken to improve the embryo selection criteria. In addition, to further reduce the risk of twins, the proportion of eSET transfers needs to be enlarged. Tiitinen *et al.* (2003) reported an increase over the years in the proportion of eSET in their IVF/ICSI programme from 10% to 56%, while maintaining comparable PRs. The same applies for Gerris *et al.*, who reported an increase of eSET from 9.8% in 1998 to 23.6% in the year 2001(Gerris *et al.*, 2002). This increase was achieved by liberalization of the criteria for application of eSET. However,

the twin PR in both clinics (ranging from 7.5% to 13.5%) (Tiitinen *et al.*, 2003; Gerris *et al.*, 2004) remained above the twin PR after a spontaneous pregnancy (ranging from 1 to 1.5%) (Ghai and Vidyasagar, 1988; Dhont, 2001). In the present study, 19% of the patients eligible for the eSET policy received one embryo. In the DET group however, a high twin PR (23%) was found in spite of "suboptimal" quality embryos. Apparently our embryo selection criteria for eSET are still too strict. Instead of gradually liberating the criteria for eSET, we decided to start a prospective randomized study in which consenting patients will be allocated by lot for the transfer of either one or two embryos, irrespective of the presence or absence of a top quality embryo. This study will provide more insight into whether we should perform SET in every patient, or how the selection of the embryos and the patients suitable for SET can be improved.

In conclusion, eSET in a selected group of patients (younger than 38 years with at least one good quality embryo) can be performed not only in the first two treatment cycles, but also in the third cycle while maintaining a PR comparable to DET. In this way the proportion of eSET in the total IVF programme can be increased, which will result in a further decline in the twin PR.

In unselected patients, elective single embryo transfer prevents all multiples, but results in significantly lower pregnancy rates as compared with double embryo transfer; a randomized controlled trial

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Abstract

BACKGROUND: Elective single embryo transfer (eSET) in a selected group of patients (i.e. young patients with at least one good quality embryo) reduces the number of multiple pregnancies in an IVF programme. However, the reduced overall multiple pregnancy rate (PR) is still unacceptably high. Therefore, a randomized controlled trial (RCT) was conducted comparing eSET and double embryo transfer (DET) in an unselected group of patients (i.e. irrespective of woman's age or embryo quality).

METHODS: Consenting unselected patients were randomized between eSET (RCT-eSET) (n=154) or DET (RCT-DET) (n=154). Randomization was performed just prior to the first embryo transfer, provided that at least two 2PN zygotes were available. Non-participants received our standard transfer policy (SP-eSET in a selected group of patients (n=100), otherwise SP-DET (n=122)).

RESULTS: The ongoing PR after RCT-eSET was significantly lower as compared with RCT-DET (21.4% versus 40.3%) and the twin PR was reduced from 21.0% after RCT-DET to 0% after RCT-eSET. The ongoing PRs after SP-eSET and SP-DET did not differ significantly (33.0% versus 30.3%), with an overall twin PR of 12.9%.

CONCLUSION: To avoid twin pregnancies resulting from an IVF treatment, eSET should be applied in all patients. The consequence would be a halving of the ongoing PR as compared with applying a DET policy in all patients. The transfer of one embryo in a selected group of good prognosis patients leads to a less drastic reduction in PR but maintains a twin PR of 12.9%.

Introduction

A multiple pregnancy is a serious adverse outcome of an IVF treatment (Land and Evers, 2003). In many (European) IVF centres, the standard embryo transfer policy is to transfer two embryos (Nyboe Andersen *et al.*, 2004). However, although higher order multiple pregnancies are reduced to 2% on average, the twin pregnancy rates (PRs) remain between 20 and 35% (Nyboe Andersen *et al.*, 2004). Twin pregnancies should also be considered as a serious disadvantage, not only because of the increased risks of medical and perinatal complications (ESHRE Capri Workshop, 2000; Helmerhorst *et al.*, 2004), but also because of the increased health care costs associated with enhanced pre- and postnatal care (De Sutter *et al.*, 2002; Gerris *et al.*, 2004; Lukassen *et al.*, 2004). The only way to solve this problem is to reduce the number of embryos transferred to one.

Several studies have investigated elective single embryo transfer (eSET). At least five randomized controlled trials (RCTs) have been published (Gerris *et al.*, 1999; Martikainen *et al.*, 2001; Gardner *et al.*, 2004; Thurin *et al.*, 2004; Lukassen *et al.*, 2005). In these studies, only patients at risk for a twin pregnancy were randomized between the transfer of one or two good quality embryos (double embryo transfer; DET). The selection criteria for patients at risk varied between the studies, but were based on female age (< 34 years, Gerris *et al.* (1999); < 35 years, Lukassen *et al.* (2005) and some of the patients in the study of Thurin *et al.* (2004); < 36 years, Martikainen *et al.* (2001) and some of the patients in the study by Thurin *et al.* (2004)), and the number of good quality embryos available (≥ 2 in the studies of Gerris *et al.* (1999), Thurin *et al.* (2004) and Lukassen *et al.* (2005), ≥3 in some of the patients in the study of Thurin *et al.* and ≥ 4 in one of the participating centres in the study by Martikainen *et al.* (2001)). Furthermore, no previous failed cycles were allowed in some of the studies (Gerris *et al.* (1999), Lukassen *et al.* (2005)). These studies showed that the transfer of only one good quality embryo resulted in lower PRs as compared with the transfer of two good quality embryos, although the differences were not significant in all studies. However, as PRs after eSET were acceptable and twin pregnancies were avoided, it was concluded by the authors that eSET should be the transfer policy of choice in this subgroup of patients. Furthermore, in two RCTs, it was demonstrated that the difference in PRs between SET and DET can be overcome by performing one additional SET cycle (Lukassen *et al.*, 2005) or a frozen embryo transfer cycle if frozen embryos from the fresh cycle were available (Thurin *et al.*, 2004).

Besides the RCTs mentioned, several observational studies have been published (Tiitinen *et al.*, 2001; Gerris *et al.*, 2002; Van Montfoort *et al.*, 2005) in which the results of eSET applied in a selected group of good prognosis patients (20-30% of the IVF population) were compared with the results of DET applied in the remaining group of patients. These studies concluded that eSET and DET resulted in comparable PRs and that the

overall twin PR in an IVF programme could be reduced considerably. In the most recent studies, 55-60% of all patients was offered eSET, while DET was offered to the remaining patients (Tiitinen *et al.*, 2003; Gerris *et al.*, 2004). This resulted in an overall twin PR of ~10%. Although this rate is significantly lower than the rate obtained with a transfer policy consisting of only DET (20-35%), it is still substantially higher than the rate in spontaneous pregnancies. From the studies published so far, it can be concluded that eSET offers an acceptable PR with a low twin PR in a selected group of patients. It is unknown whether PRs remain acceptable if eSET is applied in an unselected group of patients, thereby reducing the twin PR to a value comparable with the spontaneous twin PR. Therefore, a prospective RCT was conducted where patients were assigned to either eSET or DET irrespective of their age (within the age limits applied in our IVF programme) and irrespective of whether or not a good quality embryo was available.

The primary aim of this study was to compare the PRs in both study groups. The RCT was limited to the first cycle of patients and to the transfer of fresh embryos only. A secondary aim of this study was to evaluate PRs after eSET and DET when the decision of whether to transfer one or two embryos was based on female age (<38 years) and the presence of at least one good quality embryo.

Materials and methods

Patients and study design

From January 2002 until December 2004, patients who started their first IVF cycle in the academic hospital of Maastricht, the Netherlands, were assessed for eligibility to participate in the study. Patients applying for preimplantation genetic diagnosis (PGD), patients requiring the transfer of only one embryo (in most cases because of medical reasons), and patients who could not be informed adequately because of a language barrier were excluded. All other patients were informed about the study, including the possibility of a lower PR after eSET and the pre- and postnatal risks of twins. Eligible patients choosing not to participate in the RCT were offered our standard transfer policy (see below).

Consenting patients had to have normal fertilization of at least two oocytes (i.e. 2PN embryos) in order to be randomized between eSET (referred to as the RCT-eSET group) and DET (referred to as the RCT-DET group). Randomization was performed immediately prior to embryo transfer. To ensure comparability between eSET and DET with respect to female age (< 38 years or ≥ 38 years) and fertilization technique (IVF or IVF/ICSI), the patient population was stratified with respect to these four characteristics. Furthermore, to avoid confounding by fluctuations of success rates over time, the groups were subdivided into smaller groups to ensure an equal distribution of eSET and DET over a time-period (an

average of 3 months). By varying the size of these subgroups (ranging from 8 to 14) and by using a non-transparent box containing the sealed opaque envelopes, the randomization procedure was blinded. The laboratory personnel performing the randomization were unaware of the size of the subgroups.
After transfer, patients were informed about the number of embryos transferred. Any subsequent IVF or IVF/ICSI cycle and all transfer cycles of cryopreserved embryos were not a part of the RCT. In these cycles our standard transfer policy was applied. The standard transfer policy in our clinic consisted of the transfer of a single embryo when female age was <38 years and at least one good-quality embryo (see below) was available. Otherwise, two embryos were transferred. In order to compensate for any possible disadvantage due to a lower PR in the RCT-eSET group of the study, these patients were offered a fourth IVF or IVF/ICSI cycle free of charge if pregnancy was not achieved in the first three cycles which, as a rule, were covered by the national health system or by private insurance companies in the Netherlands.
To address the secondary aim of our study, data from patients who received the standard transfer policy in their first treatment cycle were used. This group was composed of all patients eligible for the study, but not willing to participate and the patients not eligible for the study because of a language barrier (non-participants group). Furthermore, in order to compare eSET (referred to as the standard policy eSET group, SP-eSET) with DET (SP-DET group), at least two normally fertilized oocytes (2PN zygotes) had to be present. The study was approved by the Institutional Ethical Board of the academic hospital of Maastricht and all participating patients in the RCT signed an informed consent. The non-participating patients signed an informed consent for the use of their data.

Sample size calculation
To calculate sample size for the RCT part of the study, data from a previous period in our IVF clinic, in which DET was our standard policy, were used. An ongoing PR of 29% was achieved after DET. Assuming a similar ongoing PR for the DET group in the study and considering an ongoing PR in the eSET group of < 15% as clinically unacceptable, the required sample size was 150 cycles in both the eSET and DET study group with a power of 80% and an α of 0.05.

Ovarian stimulation protocol
Patients were downregulated with 0.1 mg triptorelin daily s.c. (Decapeptyl; Ferring B.V., Hoofddorp, The Netherlands) according to the long protocol. To stimulate multiple follicular development, recombinant FSH (Puregon, Organon, Oss, The Netherlands) was used. Follicle growth was monitored by ultrasound and 5000 IU of hCG (Pregnyl, Organon, Oss, The Netherlands) was administered as soon as at least three follicles were ≥ 18 mm. Ultrasound-

guided oocyte retrieval was performed 36 h after hCG administration. The luteal phase was supported by progesterone (Progestan, Organon) 200 mg three times daily intravaginally, starting at the day of ovum pick-up and continued for 14-16 days. In case of a pregnancy, progesterone was continued for another 3 weeks.

Culture procedure, embryo quality assessment and transfer policy
IVF, ICSI and embryo culture procedures have been described in detail earlier (Dumoulin *et al.*, 2000). For each embryo originating from a normally fertilized oocyte, an embryo score was calculated on the basis of morphological grade (1-4, with grade 4 being the best grade), number of blastomeres and presence or absence of multinucleated blastomeres (MNBs) (Van Montfoort *et al.*, 2005). Embryos that had reached the 4- or 5-cell stage on day 2, or the 8-cell stage on day 3, in combination with having the best morphological grade (regular, even sized blastomeres with less than 20% fragmentation) and an absence of MNBs were classified as good quality embryos (Van Montfoort *et al.*, 2005). Embryos were transferred on day 2 after ovum pick-up or, in a minority of cases, for reasons of convenience, on day 3. In all cases, including those in the RCT study, embryos with the highest embryo score were transferred.
Cryopreservation of supernumerary embryos was performed on the morning of the third day after ovum pick-up if one or more embryos had reached the 8-cell stage, and if they were of good morphological quality.

Outcome variables
Primary outcome variables were ongoing PR and twin PR at 10 weeks after ovum pick-up (12 weeks gestational age). An hCG pregnancy test with a detection limit of 50 IU/l in urine was performed 14-16 days after embryo transfer, and patients with a positive test had an ultrasound examination 3 weeks later. An ongoing pregnancy was defined as the presence of at least one intrauterine gestational sac with fetal heartbeat on ultrasound at 7 weeks gestation, and no report of pregnancy loss when patients were contacted at 12 weeks gestation.

Statistics
Analysis of variance (ANOVA) with Tukey's multiple test procedure was used to compare the continuous variables and the χ^2 test with Bonferroni correction was used for binary variables. A P value < 0.05 was considered significant.

Results

Of the 807 patients who visited the clinic for their first IVF or IVF/ICSI cycle in the period from January 2002 until December 2004, 133 (16.5%) patients did not meet the inclusion criteria (PGD (n = 72), eSET for medical reasons (n = 42) or language barrier (n = 19)). Furthermore, 53 (6.6%) patients refrained from IVF treatment or achieved a spontaneous pregnancy while waiting for the start of their first IVF cycle. Of the 621 eligible patients, 348 (56%) agreed to participate in the RCT part of the study. Of these, 40 patients (11%) could not be randomized because of fertilization failure or because only one embryo was available. The remaining 308 patients were randomized immediately prior to embryo transfer: 154 patients received one embryo and 154 received two (Figure 1). In 123 (80%) patients in the SET group and 116 patients (75%) in the DET group, the transfer was performed on day 2. The remaining patients received their embryos on day 3.

Patient and cycle characteristics were comparable between the two study groups of the RCT (Table I). When the clinical outcomes of the eSET and DET group of the RCT were compared, the percentage of positive pregnancy tests after transfer of fresh embryos differed significantly between eSET and DET (33.1 versus 47.4%, respectively) (Table II). In addition, the abortion rate was significantly higher after eSET as compared with DET (35.3 versus 15.1%), resulting in a doubling of the ongoing PR after DET as compared with eSET (40.3 versus 21.4%). The twin PR after eSET was reduced to 0%, whereas 21.0% of the ongoing pregnancies after DET were twin pregnancies (Table II).

In the non-participants group (n=292, composed of 273 patients who declined to participate in the RCT and 19 otherwise eligible patients with a language barrier), in 70 patients the standard transfer policy could not be applied because of the following reasons: a) cancellation of the cycle before ovum pick-up (n=28), b) no fresh embryo transfer and cryopreservation of all embryos was performed because of ovarian hyperstimulation syndrome (OHSS) (n=3), c) total fertilization failure was found (n=11) or d) only one embryo was obtained and transferred (compulsory SET) (n=28). The remaining 222 patients had at least two fertilized embryos and were suitable for a comparison between eSET and DET according to our standard transfer policy (SP-eSET and SP-DET). SP-eSET was applied in 45% of the patients (Table III). Patient characteristics from the non-participants group were similar to those of the participants in the RCT study except for the mean age (Table I). The ongoing PRs in the SP-eSET and SP-DET groups were 33.0% and 30.3%, respectively (Table III). The overall ongoing PR with the standard transfer policy was 31.5% and the overall twin PR was 12.9%.

Discussion

During the last few years eSET has become more and more accepted as the solution for the high multiple PR after IVF and IVF/ICSI. Until now, eSET was only applied in a selected group of patients. In this study, an RCT was performed in the first cycle of an unselected group of patients, i.e. irrespective of female age (within the age limits applied in our IVF programme) and irrespective of the availability of a good quality embryo. The transfer of one embryo in this unselected group resulted in a significantly lower PR (21.4%) than the transfer of two embryos (40.3%). This tendency is also seen in previously performed RCTs, which were conducted in a subset of good prognosis patients. In these studies, the ongoing PRs after eSET and DET were 38.5 versus 74.0% (Gerris *et al.*, 1999) and 28.5 versus 44.1% (Thurin *et al.*, 2004), and the live birth rates after eSET and DET were 29.7 versus 40.0% (Martikainen *et al.*, 2001) and 26 versus 36% (Lukassen *et al.*, 2005), respectively. In all the aforementioned studies, in selected, good prognosis patients as well as in non-selected patients (present study), the transfer of two embryos leads to higher PRs than the transfer of one embryo.

In contrast to the markedly reduced ongoing PR found in our study after eSET compared with DET in an unselected group of patients, the ongoing PR between eSET applied in a subset of good prognosis patients and DET applied in the remaining patients (33.0% after SP-eSET versus 30.3% after SP-DET) was comparable. This is in agreement with the observational studies described in literature, obtaining PRs of 34 versus 37% (Tiitinen *et al.*, 2003) and 40.3 versus 40.4% (Gerris *et al.*, 2004), respectively, after applying eSET in a subgroup of good prognosis patients and DET in the remaining patients. The twin PR in the overall IVF programme when applying our standard embryo transfer policy was 12.9% in our study, which is comparable with the findings of other studies (7.5%, (Tiitinen *et al.*, 2003) and 13.5%, (Gerris *et al.*, 2004)).

The ongoing PR obtained with our standard transfer policy (eSET in selected patients) is higher than with a policy of transferring one embryo in all patients (31.5 versus 21.4%). This is, at least for patients < 36 years, confirmed by the data from Debrock and co-workers, who compared PRs before and after implementation of the new legislation on embryo transfer in Belgium (Debrock *et al.*, 2005). Before implementation, one embryo was transferred just in case it was a good quality embryo.

After implementation, in patients <36 years, no selection based on embryo quality was made for eSET, which means that all patients <36 years received one embryo. The clinical PR decreased non-significantly from 41.0% before implementation to 35.1% after implementation.

Of all eligible patients in our study, 46% were randomized in the RCT part of the study, comparing favourably with the participation rate in other studies

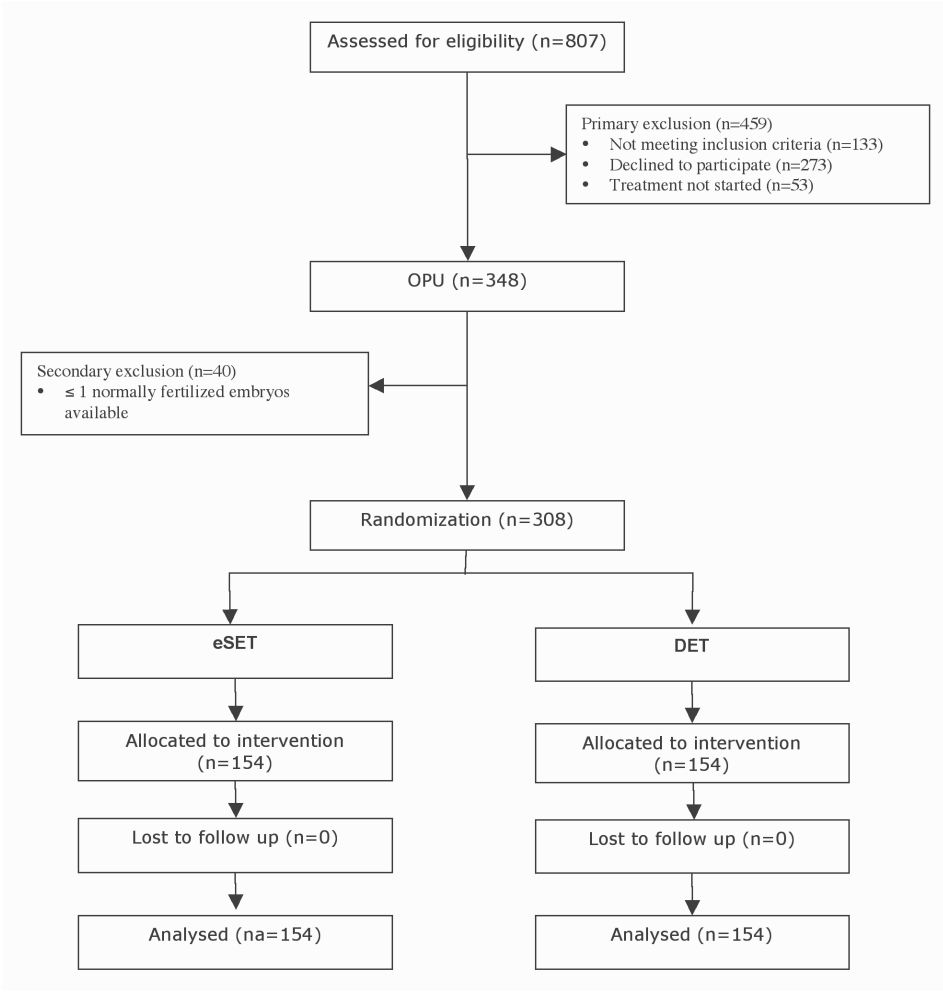

Figure 1: Flowchart of patient selection for the RCT part f the study

Table I: Patients' and cycle characteristics of the study subjects (RCT-eSET and RCT-DET) and the non-participants in the first cycle.

	RCT-eSET (n=154)	RCT-DET (n=154)	Non-participants (n=222)
Patients' characteristics			
Mean female age ± SD	32.7 ± 3.3[a]	32.4 ± 3.3[a]	34.0 ± 3.9[b]
No. of patients ≥ 38 years (%)	8 (5.2)[a]	6 (3.8)[a]	47 (21.2)[b]
Duration of subfertility ± SD	3.3 ± 1.8	3.3 ± 2.1	3.5 ± 2.8
Cause of subfertility (%)			
Tubal factor	24 (15.6)	28 (18.2)	38 (17.1)
Male factor	85 (55.2)	87 (56.5)	124 (55.9)
Unknown	34 (22.1)	37 (24.0)	45 (20.2)
Other	11 (7.1)[a]	2 (1.3)[b]	15 (6.8)[a]
Cycle characteristics			
No. of ICSI cycles (%)	88 (57.1)	89 (57.8)	126 (56.8)
No. of IVF cycles (%)	66 (42.9)	65 (42.2)	96 (43.2)
Mean n°. of oocytes per retrieval ± SD	8.9 ± 4.3	9.6 ± 4.7	9.2 ± 4.6
Fertilization rate ± SD	72.5 ± 22.2	68.4 ± 21.2	73.2 ± 20.1
Rate of embryos with ≥ 4 blastomeres on day 2 per retrieval ± SD	72.9 ± 28.7	71.3 ± 29.5	66.6 ± 29.6
Mean embryo score on day 2 ± SD	8.4 ± 2.4	8.6 ± 2.4	8.2 ± 2.6
No. of cycles with at least one good quality embryo (%)	63 (40.9)	66 (42.9)	100 (45.0)

[a,b] numbers with different superscripts within one row are significantly different (ANOVA with Tukey's multiple test procedure for continuous variables and χ^2 test with Bonferroni correction for binary variables, $P < 0.05$)

Table II: Clinical outcomes in the first IVF treatment cycle of the groups randomized for eSET (RCT-eSET) and DET (RCT-DET)

	RCT-eSET (n=154)	95% CI (%)	RCT-DET (n=154)	95% CI (%)
Positive hCG test (% per ET)	51 (33.1%)[a]	25.7 – 40.5	73 (47.4%)[b]	39.5 – 55.3
Abortion <13 weeks (% per positive hCG test)	18 (35.3%)[a]	22.2 – 48.4	11 (15.1%)[b]	6.9 – 23.3
Ongoing pregnancy at 12 weeks (% per ET)	33 (21.4%)[a]	14.9 – 27.9	62 (40.3%)[b]	32.5 – 48.0
Twin pregnancy (% per ongoing pregnancy)	0 (0%)[a]	-	13 (21.0%)[b]	10.8 – 31.1
No. of cycles with cryopreservation	73 (47.4%)	39.5 – 55.3	58 (37.7%)	30.0 – 45.3

[a,b] numbers with different superscripts within one row are significantly different (χ^2 test, $P < 0.05$)

Table III: Clinical outcome in the first IVF treatment cycle in non-participating patients treated according to the standard embryo transfer policy (n=222) with eSET (SP-eSET) or DET (SP-DET)

	SP-eSET (n=100)	95% CI (%)	SP-DET (n=122)	95% CI (%)
Transfer of fresh embryos				
Positive hCG (% per ET)	45 (45.0)	35.2 – 54.8	43 (35.2)	26.7 – 43.7
Abortion <13 weeks (% per positive hCG test)	12 (26.7)	13.7 – 39.6	6 (14.0)	3.6 – 24.3
Ongoing pregnancy at 12 weeks (% per ET)	33 (33.0)	23.8 – 42.2	37 (30.3)	22.2 – 38.5
Twin pregnancy (% per ongoing pregnancy)	1 (3.0)[a]	0 – 8.8	8 (21.6)[b]	8.4 – 34.9
No. of cycles with cryopreservation	56 (56.0)[a]	46.3 – 65.7	32 (26.2)[b]	18.4 – 34.0
Transfer of fresh and frozen embryos				
Ongoing pregnancy at 12 weeks (% per ET)	42 (42.0)	32.3 – 51.7	40 (32.8)	24.5 – 41.1
Twin pregnancy (% per ongoing pregnancy)	4 (9.5)	0.6 – 18.4	8 (20.0)	7.6 – 32.4

[a,b] numbers with different superscripts within one row are significantly different (χ^2 test, $P < 0.05$)

(11%, (Martikainen *et al.*, 2001); 25%, (Thurin *et al.*, 2004); 33%, (Lukassen *et al.*, 2005) and 39%, (Gerris *et al.*, 1999)). The higher participation rate in our study might explain the slightly lower PRs compared with the other RCTs described in literature. A high rate of participation will diminish selection bias. To evaluate selection bias in our study, the characteristics of the study population and the non-participants were compared. It was shown that the study population reflected the total IVF population with the exception of the percentage of patients ≥ 38 years, which was 4.5% in the study population and 21.2% in the non-participants. This can be explained by the fact that patients ≥ 38 years often considered their chance for pregnancy to be low and requested the standard embryo transfer policy, in which they would receive two embryos.

As our study shows, after the transfer of only a single embryo, more good quality embryos are left for cryopreservation. If the transfer of cryopreserved embryos (for practical reasons not a part of this RCT and therefore performed according to the standard transfer policy) was included in the results, the probability of a pregnancy after the first ovum pick-up increased. The overall PR was nevertheless still significantly lower in the eSET group as compared with the DET group (29.9% versus 42.2%, respectively). As from 17 eSET and 10 DET cycles a transfer of frozen/thawed embryos has not yet been performed, cumulative ongoing PRs will increase. The importance of cryopreserved embryos in eSET has already been stressed by other authors (Tiitinen *et al.*, 2001; Thurin *et al.*, 2004).

A remarkable finding in our study is the high abortion rate after eSET (35% in the RCT-eSET group and 27% in the SP-eSET group). It is known that in the first weeks after implantation 15-20% of all pregnancies are lost, both in spontaneous pregnancies and in pregnancies conceived after assisted reproduction (Tummers *et al.*, 2003; De Neubourg *et al.*, 2004). Theoretically, the difference in abortion rate found between eSET and DET might be explained by vanishing twins in the DET group, which are continuing as a singleton pregnancy and therefore not recorded as an abortion. Since it has been shown that in pregnancies with two gestational sacs ~30% result in a singleton pregnancy, only part of the difference in abortion rate between eSET and DET can be explained by vanishing twins (Landy and Keith, 1998). Winter *et al.* (2002) found that pregnancy loss before 6-7 weeks of gestation was related to poor embryo quality. However, in our study, the high abortion rate after eSET was found not only in the eSET group of the RCT part (in which 31% of the pregnancies developed after transfer of poor quality embryos) but also in the eSET group of our standard transfer policy group (in which eSET was performed with good quality embryos only). In addition, in the RCT-eSET group, 33% of the pregnancies achieved after the transfer of a poor quality embryo and 38% of the pregnancies achieved after the transfer of a good quality embryo resulted in an abortion. This indicates that the abortion rate in our

study was not related to poor embryo quality.
Whether eSET or DET is preferable depends not only on ongoing PRs and twin PRs, but also on several other factors, such as patients' preferences and the health care system in a particular country. Patients should be counselled thoroughly about PRs in the different transfer policies and about the risks associated with multiple pregnancies. Patients' attitudes towards eSET was shown to be positively adjusted in countries where new legislation regarding the embryo transfer was implemented, stating that eSET should be the routine procedure in at least the first cycle of young patients (Ombelet, 2004; Thurin *et al.*, 2004). Finally, the extent of reimbursement of costs will influence the acceptance of eSET. Therefore, as an integral part of the present study the cost-effectiveness of eSET compared with DET was analysed (Fiddelers *et al.*, 2005).
In conclusion, our study shows that applying eSET in the first cycle of an unselected group of patients will lead to a twin PR of 0%. The price to be paid is a reduction of the ongoing PR to approximately half of that obtained after DET. The transfer of one embryo in a selected group of good prognosis patients leads to a less drastic reduction in PR but maintains a twin PR of 12.9%.

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CHAPTER 4

Single versus double embryo transfer: cost-effectiveness analysis alongside a randomized clinical trial

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Abstract

BACKGROUND: Twin pregnancies after IVF are still frequent and are considered high-risk pregnancies leading to high costs. Transferring one embryo can reduce the twin pregnancy rate (PR). We compared cost-effectiveness of one fresh cycle elective single embryo transfer (eSET) versus one fresh cycle double embryo transfer (DET) in an unselected patient population.

METHODS: Patients starting their first IVF cycle were randomized between eSET and DET. Societal costs per couple were determined empirically, from hormonal stimulation up to 42 weeks after embryo transfer. An incremental cost-effectiveness ratio (ICER) was calculated, representing additional costs per successful pregnancy.

RESULTS: Successful PRs were 20.8% for eSET and 39.6% for DET. Societal costs per couple were significantly lower after eSET (€7334) compared to DET (€10924). The ICER of DET compared to eSET was €19096, meaning that each additional successful pregnancy in the DET group will cost €19096 extra.

CONCLUSIONS: One cycle eSET was less expensive, but also less effective compared to one cycle DET. It depends on society's willingness to pay for one extra successful pregnancy, whether one cycle DET is preferred from a cost-effectiveness point of view.

Introduction

One of the most important complications of infertility treatments is the high percentage of multiple pregnancies. In 2001, of all IVF pregnancies in Europe, 24.0% were twin pregnancies (Andersen *et al.*, 2005) compared with 1.2% twin pregnancies after natural conception (ESHRE Capri Workshop Group, 2000). Twin pregnancies are considered high-risk pregnancies for both mother and infants because of the relative high incidence in obstetric, perinatal and neonatal complications, which at the same time lead to high health-care costs. Up till now, five studies have been performed on the cost-effectiveness of elective single embryo transfer (eSET) versus double embryo transfer (DET) (Wolner-Hanssen and Rydhstroem, 1998; De Sutter *et al.*, 2002; Gerris *et al.*, 2004; Lukassen *et al.*, 2005; Thurin Kjellberg *et al.*, 2006).

One study from Sweden used a hypothetical take-home baby rate after SET and empirical pregnancy rates (PRs) after DET to compare costs per successful pregnancy (Wolner-Hanssen and Rydhstroem, 1998). Another study from Belgium (De Sutter *et al.*, 2002) used a Markov model to estimate cost-effectiveness of eSET versus DET, in which pregnancy probabilities for eSET and DET were based on results from other studies, and costs were based on data from a local hospital. In a study by Gerris *et al.* (2004), also from Belgium, the cost-effectiveness of eSET and DET was compared by offering patients the choice of SET, in case one good quality embryo was available, and DET, irrespective of embryo quality. Furthermore, in a Dutch study by Lukassen *et al.* (2005), the cost-effectiveness of two cycles eSET was compared with one cycle DET after randomization, in case two embryos were available of which one was of good quality. Finally, in a Scandinavian study by Thurin Kjellberg *et al.* (2006), the cost-effectiveness of one cycle eSET and one cycle DET was compared, in case two good quality embryos were available in patients < 36 years of age before randomization for eSET or DET. In a proportion of the patients, three good quality embryos had to be available in patients < 35 years of age.

What the former studies have in common is that eSET was performed in a sub-sample of good prognosis patients. A radical approach to reduce the twin PR would be to apply eSET in all patients, irrespective of their age and whether or not a good quality embryo is present. We have performed a randomized clinial trial (RCT), in which couples were randomized to having either eSET or DET in their first IVF treatment cycle, irrespective of age and embryo quality (Van Montfoort *et al.*, 2006). Alongside the RCT, a cost-effectiveness analysis was performed. The aim of this article is to compare the costs and cost-effectiveness of one fresh cycle eSET and one fresh cycle DET in an unselected sample of patients.

Materials and methods

Study design

From January 2002 to December 2004, all couples who entered our IVF programme had their eligibility assessed for participation in this study (Van Montfoort *et al.*, 2006); 308 couples who started their first IVF cycle were included in the study. Provided that at least two embryos (2PN) were available, couples were randomized between eSET and DET, irrespective of age and embryo quality. For a detailed overview of the patients and study design, see Van Montfoort *et al.* (2006).

Cost analysis

The cost analysis was performed from the societal perspective, according to the Dutch guidelines for cost calculations, and included health care costs and costs outside the health care sector (Oostenbrink *et al.*, 2002; Oostenbrink *et al.*, 2004). Health care costs consisted of hospital costs, and other health care costs related to the IVF procedure and resulting pregnancies. Hospital costs consisted of personnel costs, costs of material, costs of equipment, medication and overhead. Other health care costs included costs of visits to a General Practitioner (GP) or midwife care. Costs outside the healthcare system consisted of productivity costs and out of pocket costs for the couples (such as travel costs and over-the-counter-medication) associated with the IVF treatment cycle and resulting pregnancies. Costs were determined empirically for each couple entering the study from the start of the first IVF treatment cycle (i.e. hormonal stimulation) up to 42 weeks after embryo transfer (ET). For the subgroup of pregnant patients, this means that costs were determined up to 4 weeks after giving birth. For couples with a negative pregnancy test, we assumed no costs related to the first IVF treatment cycle were made after the first IVF cycle. Costs were calculated by multiplying volumes of use with unit prices. Subtotal costs were determined for hospital costs, other health care costs and costs outside health care made during the IVF treatment cycle, pregnancy as well as delivery and up to 4 weeks after delivery. All costs were determined for the year 2003.

Volumes of use

Cost diaries were used to determine volumes of use during the IVF treatment cycle, during pregnancy (from 13 weeks onwards, i.e. ongoing pregnancy), delivery and up to four weeks after delivery. In these diaries we asked for visits to a GP or midwife, for hospital visits, for hospital admissions, for over-the-counter medication, for the distance to several health care provisions, for absence of work, and other costs related to the IVF treatment cycle, pregnancy, or post-natal period (including delivery). The couples completed each cost diary prospectively for four successive weeks. The first cost diary was used to determine costs during the IVF treatment cycle,

from hormonal stimulation (2 weeks before ET) until pregnancy test (2 weeks after ET), to reflect care directly related to IVF. For patients with a positive pregnancy test, no cost diary was administered to determine volumes of care until 13 weeks of pregnancy. Therefore, we assumed that they had one hospital visit, including an ultrasound at seven weeks of pregnancy. With respect to patients who miscarried before 13 weeks of pregnancy, we assumed they had another hospital visit including an ultrasound at the hospital (a total of two hospital visits and two ultrasounds). To determine volumes of use during pregnancy from 13 weeks onwards, patients with a singleton pregnancy were asked to complete two cost diaries for two randomly selected periods, whereas patients with a twin pregnancy completed three cost diaries for three randomly selected periods. Patients who were pregnant with twins were asked to complete three instead of two cost diaries, because only 20% of the pregnant patients in the DET strategy had a twin pregnancy (Van Montfoort *et al.*, 2006). In that way, we still received sufficient information of medical consumption and other costs during pregnancy. To calculate the total costs per patient, the costs per period were added, assuming that the subgroup of patients (13 – 26 patients per period, of whom two to five patients were pregnant with twins) was representative for the total group of pregnant patients. The last cost diary was used to determine volumes of use of delivery and after delivery, starting from delivery up to 4 weeks later.

Unit prices

Unit prices were obtained from the hospital financial department, by cost price calculation and by using guideline prices. All hospital costs during the IVF treatment cycle, except the costs of the laboratory phase were calculated using unit prices by the financial department of the University Hospital, Maastricht. These unit prices include the general overhead costs. Unit prices were linked to the volumes of use obtained from the cost diaries to determine the mean hospital costs per couple. The cost of the laboratory phase was determined by a detailed cost price calculation (Oostenbrink *et al.*, 2004). First, the total costs for the year 2003 of all equipment used for IVF (depreciation, interest, and maintenance costs of invested capital) were determined. Furthermore, material costs related to IVF (such as needles and chemicals) and personnel costs were determined for the year 2003. The total direct costs were raised with 35% to include the general overhead of the hospital (Oostenbrink *et al.*, 2004). To calculate the mean laboratory costs per IVF cycle per couple, total costs were divided by the number of ovum pick ups (OPUs) performed in 1 year.

For calculation of costs outside the hospital, guideline prices were used. Productivity losses were valued by using the friction cost method, which assumes that within a productivity process, everybody is replaceable. Productivity costs only appear during the friction period which is defined as the average period that a vacancy exists in a society (Oostenbrink *et al.*, 2004; Brouwer and Koopmanschap, 2005).

Cost-effectiveness analysis
A cost-effectiveness analysis was performed based on the incremental costs per extra successful pregnancy. A successful pregnancy was defined as a pregnancy resulting in at least one live-born child. The incremental cost effectiveness ratio (ICER) was calculated by using the following formula: (mean total costs DET – mean total costs eSET) / (proportion of successful pregnancies DET – proportion of successful pregnancies eSET).

Sensitivity analyses
Recently, an integrative study was performed in which the cost-effectiveness of several IVF treatment strategies in the Netherlands was determined, based on six IVF-related studies financed by the Dutch Organisation for Health Research and Development (ZonMw). For this, mean unit prices were determined, based on unit price information from all participating centres (ZonMw, 2005). These 'Dutch' unit prices were available for a limited number of cost units. First, a sensitivity analysis was performed in which these 'Dutch' unit prices were used instead of the Maastricht unit prices, to investigate the influence on the cost-effectiveness of eSET versus DET. Second, a sensitivity analysis was performed in which the costs of IVF medication were set on the lowest and highest values (€1046 and €1550). Third, as costs from 5 to 12 weeks of pregnancy were based on an assumption, sensitivity analyses were performed in which a 'low-care' and 'high-care' assumption was made for estimation of the costs of that period. In the 'low-care' assumption, only half of the pregnant patients had one visit including an ultrasound and only half of the patients who miscarried had one visit including an ultrasound. Furthermore, in the 'high-care' assumption, all pregnant patients had two hospital visits including an ultrasound and one visit to a GP, and all patients who miscarried had two hospital visits including an ultrasound. Fourth, for the post-natal costs, cost data from Lukassen *et al.* (2004) were used, because in our study, we had information only on these costs from 29% of the pregnant patients (25% of the twin pregnancies and 31% of the singleton pregnancies) who completed the last cost diary. However, in the study of Lukassen *et al.*, only hospital costs were calculated. Therefore, only these costs were changed in the sensitivity analysis. Fifth, hospital admissions during the IVF treatment cycle were excluded from the cost calculation, because the difference in costs of hospital admissions during the IVF treatment cycle between eSET and DET could not be attributed to the ET policy. Sixth, the unit price of a hospital admission day was changed into guideline prices according to Oostenbrink *et al.* (2004) (€337 and €476 per day for general hospital and university hospital, respectively). Finally, as the cost analysis was based on couples, in a secondary analysis, costs were determined for female patients only, i.e. those actually undergoing the first IVF treatment cycle, excluding male partners from the cost analysis.

Statistical analysis

The statistical analysis was performed according to the intention-to-treat principle. A Missing Value Analysis (Statistics Package for Social Sciences, (SPSS)) was performed to estimate missing items in the cost diaries. To estimate 'missing' cost diaries, the overall groups' mean of eSET and DET was calculated, and resulting values were imputed (mean substitution). To quantify uncertainty around the cost-differences between eSET and DET, subtotal IVF costs, pregnancy costs and post-natal costs, and total societal costs for eSET and DET were bootstrapped. The bootstrap method estimates the sampling distribution of a statistic through a large number of simulations, based on sampling with replacement from the original data (Briggs *et al.*, 1997). The results based on 1000 bootstrap replications of the costs for eSET and DET were used to calculate 95% uncertainty intervals (UI) around the cost-differences, based on the 2.5th and 97.5th percentiles. Also, bootstrap simulations were conducted to get insight in the uncertainty around the ICER (Efron and Tobshirani, 1993), yielding information about the joint distribution of the cost and effect differences. The results based on 1000 successive bootstrap replications were presented in a cost-effectiveness plane. The horizontal axis of the cost-effectiveness plane shows the difference in effect between the groups and the vertical axis shows the difference in costs. The choice of a treatment strategy depends on the maximum amount of money that the society is prepared to pay for a gain in effectiveness, which is called the ceiling ratio. A ceiling ratio can vary from zero to infinity and can be represented by an imaginary straight line through the origin of the cost-effectiveness plane, starting with a slope of zero (representing a ceiling ratio of zero) and increasing the slope to 90 degrees (representing a ceiling ratio of infinity). For any given ceiling ratio, the proportion of bootstrap replications with an ICER equal to or lower than that particular ceiling ratio was determined. The probability that an intervention is cost-effective in relation to different values of the ceiling ratio was reflected in a cost-effectiveness acceptability curve (Van Hout *et al.*, 1994). Also, a cost-effectiveness acceptability curve was derived using the results of the sensitivity analysis in which postnatal cost data of Lukassen *et al.* (2004) were used. All analyses were performed in SPSS 12.0 with the exception of the bootstrap analysis which was performed in Excel 2000.

Results

In table I, baseline socio-economic characteristics are given for both the groups. All characteristics were comparable for eSET and DET. Other patients' and cycle characteristics such as female age and fertilization rate were also comparable for both groups. For a detailed overview of the latter, we refer to our previous publication (Van Montfoort *et al.*, 2006).

Effectiveness
Table II shows the clinical outcomes of one cycle eSET versus one cycle DET. After one cycle eSET and one cycle DET, 33.1 and 47.4% of the patients had a positive pregnancy test, respectively, and 21.4 and 40.3% achieved an ongoing pregnancy (more than 12 weeks of pregnancy). In both the groups, one late abortion (after 12 weeks of pregnancy) occurred, resulting in 20.8% successful pregnancies in the SET group and 39.6% in the DET group. Of the successful pregnancies, 0 and 19.6% were twins after one cycle eSET and DET, respectively (Van Montfoort *et al.*, 2006).

Cost-analysis
Table III summarizes the costs divided in IVF treatment costs, costs of pregnancy, delivery and up to 4 weeks later, resulting in the total societal costs per randomized couple. Subtotal costs of the IVF treatment cycle were €4431 for one cycle eSET and €4513 for one cycle DET. The bootstrapped difference of these costs was not statistically significant (95% UI: €-88 – €268). The cost difference was mainly caused by higher hospital costs after ET, because of costs of six hospital admissions in the DET group following OPU. In five of six patients, hospital admission occurred immediately after the OPU, before ET and randomisation. These admissions can therefore not be attributed to having DET. However, one patient was admitted because of ovarian hyperstimulation syndrome (OHSS) which occurred 9 days after ET. This could possibly be contributed to the fact that this patient was pregnant with twins, although a possible relationship between multiple pregnancy and OHSS has not been convincingly established in the literature. During pregnancy, all cost components were about twice as high in the DET group compared with the eSET group and the cost difference was mainly caused by differences in productivity costs and hospital costs. The bootstrapped difference of the costs during pregnancy was statistically significant (95% UI: €928 – €2622).

Table I: Socio-economic characteristics at baseline

	SET (n = 154)		DET (n = 154)	
	Female	Male	Female	Male
Marital state				
Married	98 (63.6)		97 (63.0)	
Not married or living	2 (1.3)		2 (1.3)	
Living together	25 (16.2)		25 (16.2)	
Information missing	29 (18.8)		30 (19.5)	
Having a housekeeper				
Yes	19 (12.3)		17 (11.0)	
No	108 (70.1)		101 (65.6)	
Information missing	27 (17.5)		36 (23.4)	
Education				
Lower education	19 (12.3)	22 (14.3)	20 (13.0)	18 (11.7)
Secondary ducation	27 (17.5)	15 (9.7)	22 (14.3)	19 (12.3)
Higher education	72 (46.8)	62 (40.3)	70 (45.5)	62 (40.3)
University	7 (4.5)	10 (6.5)	12 (7.8)	12 (7.8)
Information missing	29 (18.8)	45 (29.2)	30 (19.5)	43 (27.9)
Paid work				
Yes	110 (71.4)	105 (68.2)	107 (69.5)	112 (72.7)
No	17 (11.0)	13 (8.4)	19 (12.3)	4 (2.6)
Information missing	27 (17.5)	36 (23.4)	28 (18.2)	38 (24.7)
Voluntary work				
Yes	5 (3.2)	4 (2.6)	5 (3.2)	4 (2.6)
No	122 (79.2)	114 (74.0)	121 (78.6)	112 (72.7)
Information missing	27 (17.5)	36 (23.4)	28 (18.2)	38 (24.7)
Studying				
Yes	6 (3.9)	2 (1.3)	5 (3.2)	2 (1.3)
No	121 (82.5)	116 (75.3)	121 (78.6)	114 (74.0)
Information missing	27 (17.5)	36 (23.4)	28 (18.2)	38 (24.7)
Unemployed				
Yes	4 (2.6)	4 (2.6)	6 (3.9)	1 (0.6)
No	123 (79.9)	114 (74.0)	120 (77.9)	115 (74.7)
Information missing	27 (17.5)	36 (23.4)	28 (18.2)	38 (24.7)

Table I: continued				
Work-disabled				
Yes	7 (4.5)	3 (1.9)	5 (3.2)	1 (0.6)
No	120 (77.9)	115 (74.7)	121 (78.6)	115 (74.7)
Information missing	27 (17.5)	36 (23.4)	28 (18.2)	38 (24.7)
Housekeeping				
Yes	53 (34.4)	13 (8.4)	52 (33.8)	10 (6.5)
No	74 (48.1)	105 (68.2)	74 (48.1)	106 (68.8)
Information missing	27 (17.5)	36 (23.4)	28 (18.2)	38 (24.7)

Table II: Clinical outcomes of one cycle eSET versus one cycle DET[1]

	SET (n=154)	DET (n=154)
Positive HCG test (% per ET)	51 (33.1%)	73 (47.4%)
Abortion <13 weeks (% per positive HCG test)	18 (35.3%)	11 (15.1%)
Ongoing pregnancies (% per ET)	33 (21.4%)	62 (40.3%)
Successful pregnancies (% per ET)	32 (20.8%)	61 (39.6%)
Twin pregnancies (% per successful pregnancy)	0 (0%)	12 (19.6%)
Number of children born (% per ET)	32 (20.8%)	73 (47.4%)

[1] Adapted from Van Montfoort *et al.* (2006). Outcomes expressed per ET as eSET and DET are the comparators in this study

From delivery until 4 weeks later, the higher costs of DET compared with eSET were mainly caused by differences in hospital costs and productivity costs. The bootstrapped difference of the costs after delivery was also statistically significant (95% UI: €966 – €2637). The total societal costs per couple were €7334 for one cycle eSET compared to €10924 for one cycle DET, which was statistically significantly different (95% UI: €2060 – €5290). Most costs during the IVF treatment cycle, pregnancy and post-natal period were due to female patients (97% of the total societal costs in both the groups). The male partners were mainly responsible for the 'leave of absence' costs during the IVF treatment cycle (70%), pregnancy (27%) and from delivery up to four weeks later (100%).

Cost-effectiveness analysis
The ICER of one cycle DET compared to one cycle eSET was €19096 for the base-case analysis (table IV). This means that the incremental costs are €19096 for one extra successful pregnancy if one cycle DET will be performed instead of one cycle eSET.
Figure 1 shows the cost-effectiveness plane for the base-case analysis, in which the results based on 1000 successive bootstrap replications are given, comparing one cycle DET to one cycle eSET. The incremental cost-effectiveness acceptability curve of the base-case analysis in Figure 2 shows that until the ceiling ratio reaches €15000, the probability that one cycle DET is cost-effective is 0%, as all bootstrapped ICERs were equal to or higher than €15000. When the ceiling ratio is between €15000 and €32500, the probability that one cycle DET is cost-effective increases and the probability that one cycle eSET is most cost-effective decreases. If the ceiling ratio is above €32500, the probability that one cycle DET is most cost-effective is 100%, as all bootstrapped ICERs were equal to or lower than €32500.

Sensitivity analyses
Results of the sensitivity analyses are summarized in table IV. The ICERs of one cycle DET compared with one cycle eSET changed only marginally if several cost parameters were altered. Using post-natal hospital costs of Lukassen *et al.* (2004) in a sensitivity analysis had the largest effect on the ICER. Therefore, results from this analysis were bootstrapped, and a cost-effectiveness acceptability curve was derived. Again, 100% of the cost-effectiveness pairs are located in the quadrant where one cycle DET is more effective but more costly compared with one cycle eSET (northeast quadrant; results not graphically presented). Figure 2 (Lukassen data) shows that the cost-effectiveness acceptability curve is slightly shifted to the left compared to the base-case curve in Figure 2 meaning that the results became slightly more favourable for DET. Until the ceiling ratio reaches €12500, the probability that one cycle eSET is most cost-effective is 100%. When the ceiling ratio is between €12500 and €25000, the probability that one cycle eSET is most cost-effective decreases and the probability that one cycle DET is most cost-effective increases. If the ceiling ratio is above €25000, the probability that one cycle DET is most cost-effective is 100%. As costs of pregnancy were based on a small number of pregnant couples, we also compared our hospital costs during pregnancy with the hospital costs found in the study of Lukassen *et al.* (2004). These costs (€238 for eSET and €595 for DET) were highly comparable with the hospital costs we calculated in our study (€281 for eSET and €593 for DET). Therefore, we decided not to perform a sensitivity analysis on these costs.

Table III: Mean costs IVF treatment cycle until four weeks after delivery for all 308 patients included in the study

	Unit price (€)	eSET (n = 154)		DET (n = 154)		Bootstrapped difference in costs[b] (€) (95% UI) DET – eSET
		Volumes of use	Costs per couple[a] (€) Mean (SD)	Volumes of use	Costs per couple[a] (€) Mean (SD)	
IVF treatment cycle[d]						
Hospital costs						
Hormonal stimulation phase						
Medication[e]	1298 (1046 – 1550)	11	1298 (NA)272 (NA)	11	1298 (NA) 272 NA)	
Hospital care	272(50)					
Ovum pick-up	490 (300)	1	490 (NA)	1	490 (NA)	
Laboratory phase	1100 (350)	1	1100 (NA)	1	1100 (NA)	
Embryo transfer	260 (200)	1	260 (NA)	1	260 (NA)	
Other	NA[c]	NA	242.11 (81.46)	NA	246.18 (82.99)	
Hospital admission days	265-336 / day	0	4.38 (9.97)	0.11	49.53 (290.97)	
Subtotal			3666 (82)		3716 (317)	51 (7 – 109)
Other health care costs						
General Practitioner	20.20 / visit	0.12	2.51 (7.31)	0.13	2.60 (7.14)	
Other	NA	NA	10.73 (39.99)	NA	9.29 (34.36)	
Subtotal			13 (43)		12 (35)	-1 (-11 – 7)
Costs outside health care						
Productivitycost Sick leave	29.88 – 40.86 / hour	NA	467.56 (600.52)	NA	444.73 (518.62)	
Leave of absence	9.00 / hour	12.9	116.26 (136.45)	13.6	121.99 (130.78)	
Loss of leisure time	9.00 / hour	6.6	59.71 (157.90)	11.5	103.67 (576.97)	
Out of pocket cost	NA	NA	63.22 (41.56)	NA	73.46 (50.39)	
Informal care	9.00 / hour	3.0	26.69 (61.82)	3.0	27.01 (59.77)	

Table III: Continued

	Unit price (€)	eSET (n = 154)		DET (n = 154)		Bootstrapped difference in costs[b] (€) (95% UI) DET – eSET
		Volumes of use	Costs per couple[a] (€) Mean (SD)	Volumes of use	Costs per couple[a] (€) Mean (SD)	
Other	NA	NA	18.03 (90.86)	NA	14.56 (72.21)	
Subtotal			751 (710)		785 (780)	35 (-118 – 210)
Subtotal IVF treatment cycle			4431 (731)		4513 (880)	87 (-88 – 268)
Pregnancy (5-40 weeks)						
Hospital costs						
Ultrasound & Consultation	80.58	0.33	26.78 (38.17)	0.48	38.85 (40.53)	
Miscarriage	80.58	0.13	9.98 (26.68)	0.08	6.30 (21.74)	
Other	NA[c]	NA	120.54 (253.50)	NA	274.86 (386.38)	
Hospital admission days	265-336 / day	0.62	123.49 (340.20)	1.32	273.23 (788.79)	
Subtotal			281(563)		593 (1050)	312 (129 – 528)
Other health care costs						
General Practitioner	20.20 / visit	0.19	3.85 (8.35)	0.35	6.99 (11.69)	
Midwife care	21.43 / visit	0.97	20.77 (42.70)	1.47	31.40 (48.10)	
Other	NA	NA	65.51 (165.37)	NA	104.65 (181.92)	
Subtotal			90 (205)		143 (223)	53 (1 – 103)

Table III: Continued

	Unit price (€)	eSET (n = 154)		DET (n = 154)		Bootstrapped difference in costs[b] (€) (95% UI) DET – eSET
		Volumes of use	Costs per couple[a] (€) Mean (SD)	Volumes of use	Costs per couple[a] (€) Mean (SD)	
Costs outside health care						
Productivity cost			406.90 (868.89)			
Sickleave	29.88 – 40.86 / hour	NA		NA	1236.38 (1910.63)	
Maternity leave	29.88 – 40.86 / hour	NA		NA	725.52 (1001.49)	
Leave of absence	9.00 / hour	6.63	59.69 (320.00)	14.35	129.13 (446.19)	
Loss of leisure time	9.00 / hour	0.67	6.02 (18.06)	2.26	20.30 (59.13)	
Out of pocket cost	NA	NA	19.63 (44.06)	NA	35.86 (47.22)	
Informal care	9.00 / hour	8.54	76.83 (206.87)	18.89	170.00 (276.90)	
Other	NA	NA	85.59 (188.95)	NA	198.32 (316.25)	
Subtotal			1149 (2349)		2516 (3378)	1287 (704 865)
Subtotal pregnancy[f]			1520 (3047)		3252 (4304)	1761 (928 622)
Delivery up to four weeks after delivery						
Hospital costs						
Other	NA	NA	11.22 (44.93)	NA	28.36 (72.76)	
Hospital admission days	336-804 / day	0.56	551.76 (1228.18)	4.61	1590.51 (2826.54)	
Subtotal			563 (1245)		1619 (2852)	1067 (632 603)
Other health care costs						
General Practitioner	20.20 / visit	0.13	2.69 (7.32)	0.20	4.03 (7.98)	
Midwife care	41.99 / visit	0.20	8.41 (22.18)	0.27	11.40 (28.72)	
Other	NA[c]	NA	315.36 (789.34)	NA	419.63 (712.97)	
Subtotal			326 (806)		435 (732)	111 (-71 – 275)

Table III: Continued

	Unit price (€)	eSET (n = 154)		DET (n = 154)		Bootstrapped difference in costs[b] (€) (95% UI) DET – eSET
		Volumes of use	Costs per couple[a] (€) Mean (SD)	Volumes of use	Costs per couple[a] (€) Mean (SD)	
Costs outside health care						
Productivity cost			390.52 (846.38)			
Maternity leave	29.88 – 40.86 / hour	NA		NA	831.40 (1178.93)	
Leave of absence	9.00 / hour	2.84	25.58 (80.91)	6.28	56.50 (130.44)	
Loss of leisure time	9.00 / hour	0.53	4.79 (11.90)	2.00	17.96 (80.64)	
Out of pocket cost	NA	NA .	4.06 (10.67)	NA	7.76 (17.36)	
Informal care	9.00 / hour	5.28	47.49 (115.54)	17.23	155.07 (470.33)	
Other	NA	NA	20.50 (72.87)	NA	36.35 (119.09)	
Subtotal			493 (1049)		1105 (1649)	614 (313 – 928)
Subtotal after delivery[g]			1382 (2910)		3159 (4549)	1785 (966 2637)
Total societal costs per couple			7334 (5780)		10924 (8560)	3510 (2060 290)

[a] Cost per couple = unit price * volumes of use [b] Bootstrapped difference of costs DET - costs eSET

[c] NA means not applicable. Presentation of separate volumes and cost prices was not possible because they were constructed of several medical activities. For example, other hospital costs consist of consultations, laboratory tests, etc. Each medical activity has its own volume and cost price.

[d] Two weeks before OPU until two weeks after OPU. [e] For the costs of medication, mean costs were used per couple.

[f] Mean subtotal costs after pregnancy were €7315 for pregnant patients in the eSET group and €8210 for pregnant patients in the DET group.

[g] Mean subtotal costs after delivery were €6651 for pregnant patients in the eSET group and €7975 for pregnant patients in the DET group.

Table IV: Costs, effects and incremental cost-effectiveness ratio of one cycle DET versus one cycle eSET; results from base-case analysis and sensitivity analyses

	SET			DET			
	Mean total costs (€)	Effectiveness (%)	Mean total costs / effect (€)	Mean total costs (€)	Effectiveness (%)	Mean total costs / effect (€)	ICER (% change in ICER)
Base-case analysis	7334	20.8	35260	10924	39.6	27586	19096
'Dutch' unit prices	6312	20.8	30346	9902	39.6	25005	19096 (0)
Medication costs							
Low estimate	7082	20.8	34048	10672	39.6	26949	19096 (0)
High estimate	7586	20.8	36471	11176	39.6	28222	19096 (0)
Pregnancy costs 5-12 weeks							
Low-care assumption	7315	20.8	35168	10901	39.6	27528	19074 (-0.1)
High-care assumption	7373	20.8	35447	10970	39.6	27702	19133 (+0.2)
Postnatal hospital costs Lukassen and coworkers[a]	7204	20.8	34635	10448	39.6	26384	17255 (-9.6)
Exclusion of hospital admissions during IVF treatment cycle from cost calculation	7329	20.8	35236	10874	39.6	27460	18856 (-1.3)
Unit price of hospital admission day							
€337/day	7197	20.8	34601	10599	39.6	26765	18096 (-5.2)
€476/day	7383	20.8	35495	11156	39.6	28172	20069 (+5.1)
Only female patients in analysis	7099	20.8	34130	10567	39.6	26684	18447 (-3.4)

[a] €182 for eSET and €852 for DET (Lukassen *et al.*, 2004).

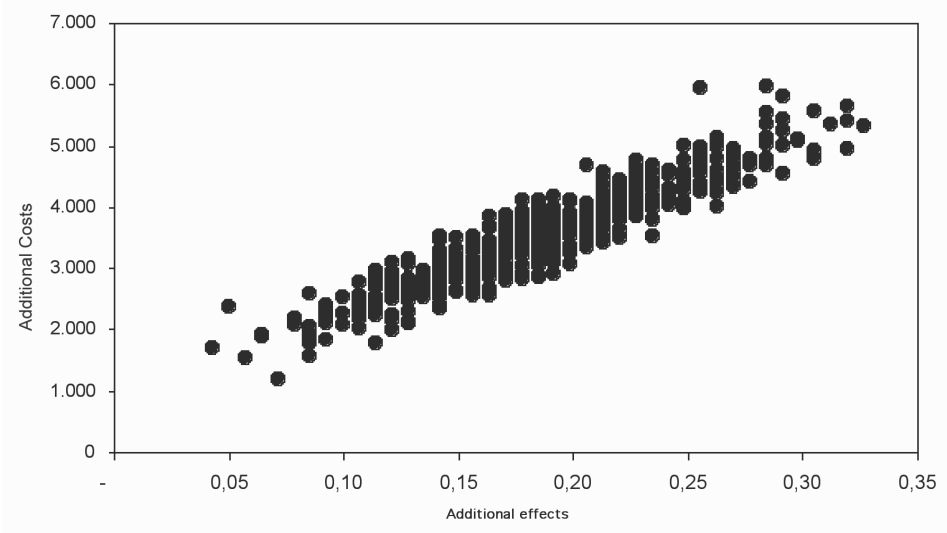

Figure 1

The cost-effectiveness plane is divided into four quadrants indicating four possible situations in relation to the additional costs and effects of one cycle DET compared to one cycle eSET (Briggs and Fenn, 1998). If the bootstrap replications are located in the northeast quadrant, DET will be more effective but also more costly compared to eSET. If they are in the southeast quadrant, DET is more effective and less costly compared to eSET, indicating dominance for DET. If they are in the southwest quadrant, DET is less costly, but also less effective compared with eSET. Finally, if they are in the northwest quadrant, DET is less effective and more costly compared to eSET, indicating inferiority for DET. The bootstrap analysis revealed that 100% of the cost-effectiveness pairs are located in the quadrant where one cycle DET is more effective but also more costly compared to one cycle eSET (northeast quadrant). Therefore, only thenortheast quadrant of the cost-effectiveness plane is shown.

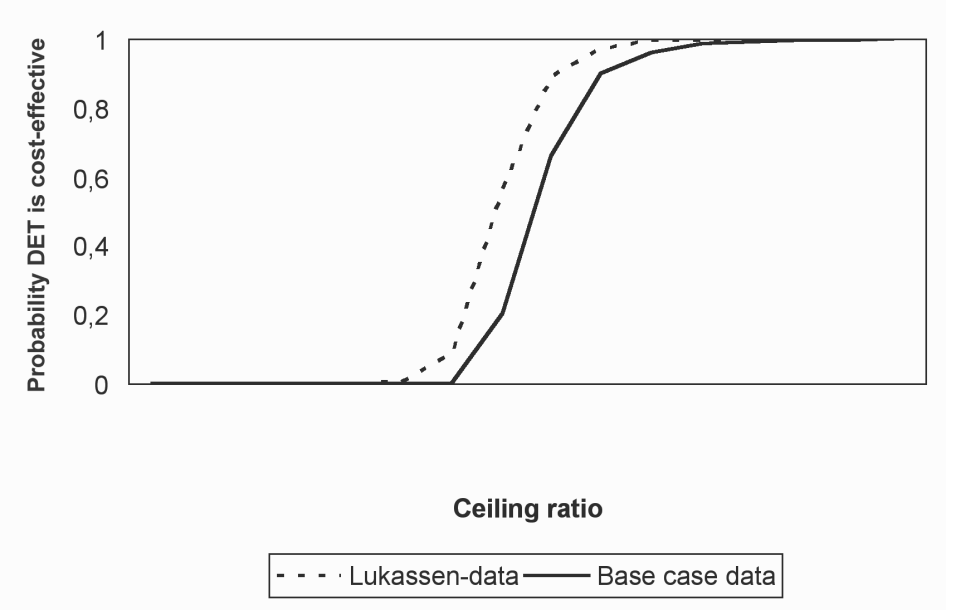

Figure 2
Cost-effectiveness acceptability curve for base-case analysis and for sensitivity analysis using postnatal costs of Lukassen *et al.* (2005)

Discussion

This study is distinct from other studies, because a cost-effectiveness analysis was performed in an unselected group of patients receiving eSET or DET, based on the couple's perspective instead of only the female patient's perspective. Socio-economic characteristics were comparable for both the groups, indicating that the difference in costs between eSET and DET (such as productivity costs and out-of-pocket costs) was not affected by differences in socio-economic characteristics. The successful PR after eSET was approximately half of that obtained after DET (20.8% after eSET versus 39.6% after DET). Total societal costs per couple were significantly lower after one cycle eSET (€7334) compared to one cycle DET (€10924).
The cost difference was mainly caused by the hospital costs after ET (40% of the cost difference) and productivity costs (32% of the cost difference). Female patients were mainly responsible for the costs in both the groups. The ICER of DET compared to that of eSET was €19096, indicating that the incremental costs are €19096 for one extra successful pregnancy if one cycle DET will be performed instead of one cycle eSET. Sensitivity analyses showed that our results were very robust for changing several cost parameters. Using post-natal hospital costs of Lukassen *et al.* (2004) caused a minor shift to the left in the cost-effectiveness acceptability curve, indicating that results became slightly more favourable for DET.
There are some limitations in the cost-effectiveness analysis that need to be addressed. First, all data with respect to the volumes of use were collected from cost diaries, because most patients dispersed to other hospitals in the south of the Netherlands during pregnancy. Because we relied on couple-reported data and made no use of official registrations, possibly under- or over-reporting may have occurred. However, it is expected that the bias resulting from it is probably equal for both the groups, so it will not influence our conclusions. Although several assumptions were made and some data were missing, our results proved to be reliable for changing several cost parameters. In addition, cost diaries have proven to be reliable (Goossens *et al.*, 2000) and are commonly used in cost-effectiveness analysis. Second, in our cost-effectiveness analysis only results of the first IVF cycle were compared.

Nowadays, there is increasing support to compare two consecutive cycles of eSET with one cycle of DET, to gain equal pregnancy probabilities. In our study, assuming that all couples without a successful pregnancy in the first eSET cycle would receive eSET for a second time, that the successful PR of a second cycle eSET would be exactly the same as in the first cycle and that the total costs would also be the same, 37.0% (32 (20.8% * 154) + 25 (20.8% * 122) / 154) of the patients would have a successful pregnancy after two cycles of eSET. The total costs would be €12728 ((€7102 * 154) + (€7102 * 122) / 154). On the basis of ICER, two cycles eSET would be

dominated by one cycle DET, because costs are higher and the effect is lower compared with one cycle DET. Furthermore, in a comprehensive cost-effectiveness analysis, several DET and eSET strategies should be compared, evaluating the full IVF procedure. For this, a Markov model is currently being developed, reflecting the 'real-world' situation as accurate as possible, taking into account cancelled cycles, availability of only one embryo (compulsory SET), declining PRs in subsequent cycles, transfers of cryopreserved embryos and treatment dropouts. In this model several eSET and DET strategies based on continued cycles are compared to determine which one is preferable.

Former studies have shown that cost differences between eSET and DET are mainly caused by high costs because of complications during twin pregnancies and deliveries and by high neonatal costs for twins in the DET group (Wolner-Hanssen and Rydhstroem, 1998; De Sutter *et al.*, 2002; Gerris *et al.*, 2004; Lukassen *et al.*, 2005; Thurin Kjellberg *et al.*, 2006). In our study, the cost difference between eSET and DET was also because of twin pregnancies and more pregnancies occurring in the DET group. Contrary to most other cost-effectiveness studies (Wolner-Hanssen and Rydhstroem, 1998; De Sutter *et al.*, 2002; Gerris *et al.*, 2004; Lukassen *et al.*, 2005) in our study, an ICER was calculated. Other studies have only calculated the mean costs per successful pregnancy (i.e. birth of at least one live-born child) for each strategy separately. According to Drummond *et al.* (2005), regarding a full economic evaluation, two criteria have to be met. First, in the study, two or more alternatives have to be compared. Second, an economic evaluation must deal with the consequences (outcome) and the costs (investment) of the treatment alternatives being compared using an incremental approach. Therefore, it is not comprehensive to only determine the mean costs per effect for each strategy separately but the incremental cost-effectiveness should be reported as well. Moreover, as these studies used a selected patient group, results cannot directly be compared to those of the present study. Only in the study of Thurin Kjellberg *et al.* (2006) an ICER was calculated. Using a societal perspective, their ICER comparing DET with SET was €91702 per extra successful pregnancy, which is about 4.5 times higher than the ICER of our study. On the basis of ICER, Thurin and co-workers concluded that DET is not cost-effective.

The choice between offering couples one cycle eSET or one cycle DET depends on what society is prepared to pay for one extra live-born child. Currently, there is no universally accepted ceiling ratio for cost-effectiveness and to date (Eichler *et al.*, 2004), most cost-effectiveness researchers only provide policymakers with cost-effectiveness acceptability curves, showing for a wide range of ceiling ratios, the probability that a particular health care commodity is cost-effective. Also within the field of IVF, there is currently no agreement on an appropriate ceiling ratio for one extra

successful pregnancy. Therefore, based on the results of this study it cannot be concluded whether DET or eSET in the first cycle is more cost-effective. On the other hand, because DET seems to be current practice the ratio from our study indicates that substituting DET by eSET in an unselected patient group would lead to cost savings but effectiveness loss as well.

A reluctance to lose a proportion of successful pregnancies would consequently indicate acceptability of the ICER we found in this study (Severens *et al.*, 2005). Nevertheless, in deciding on the preferred strategy, the long-term consequences of eSET and DET should also be considered. Inclusion of the long-term costs in the cost-effectiveness analysis, such as costs because of premature births, would probably result in a substantially higher ICER, making DET less attractive from a long-term economic point of view. However, a 'successful pregnancy' is considered an intermediate outcome measure. A commonly used outcome measure in economic evaluation is the number of quality adjusted life years (QALYs), combining the number of life years gained with the quality of that life. To obtain a balanced estimate of the long-term cost-effectiveness of eSET versus DET, both long-term costs and effects in terms of QALYs should be considered. For practical reasons, this has not been included in any study so far. Therefore, it is difficult to provide a fair estimate of the long-term cost-effectiveness.

In conclusion, our study shows that in an unselected group of patients qualifying for IVF treatment, one cycle eSET is less expensive, but also less effective compared with one cycle DET. It depends on society's willingness to pay for one extra successful pregnancy, whether one cycle eSET or one cycle DET is preferred from a cost-effectiveness point of view. The Markov model currently in development by our research group should provide insight into the 'real-world' cost-effectiveness of several SET and DET strategies.

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eSET irrespective of the availability of a good quality embryo in the first cycle only is not effective in reducing overall twin pregnancy rates

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Abstract

INTRODUCTION: In several clinics elective single embryo transfer (eSET) is applied in a selected group of patients based on age and the availability of a good quality embryo. Whether or not eSET can be applied irrespective of the presence of a good quality embryo in the first cycle, to further reduce the twin pregnancy rate (PR), remains to be elucidated.

METHODS: In patients < 38 years two transfer strategies were compared, which differed in the first cycle only: group A (n=141) received eSET irrespective of the availability of a good quality embryo, and group B (n=174) received eSET when a good quality embryo was available while otherwise they received DET (referred to as eSET/DET transfer policy). In any subsequent cycle, in both groups the eSET/DET transfer policy was applied.

RESULTS: After completion of their IVF treatment (including a maximum of three fresh cycles and the transfer of frozen-thawed embryos), comparable cumulative live birth rates (62.4% in group A and 62.6% in group B) and twin pregnancies rates (10.1% vs 13.4%) were found. However, patients in group A required significantly more fresh (2.0 vs 1.8) and frozen cycles (0.8 vs 0.5).

CONCLUSION: The transfer of one embryo in the first cycle only, in all patients < 38 years, is not an effective transfer policy for reducing the overall twin PR.

Introduction

In several randomized studies, elective Single Embryo Transfer (eSET, i.e. the transfer of one embryo in case one could also transfer two or more embryos) has proven its merits for reducing the twin pregnancy rate (PR), while obtaining acceptable overall PRs in selected patients (see Bergh (2005) for review on eSET). Based on these studies in several clinics a transfer policy was introduced in which relatively young patients with at least one or two good quality embryos received eSET and the remaining patients received double embryo transfer (DET) (referred to as eSET/DET transfer policy). This policy was applied in most studies in the first and second IVF cycle. Recently it was shown that this policy also led to acceptable results in the third IVF cycle (Van Montfoort *et al.*, 2005).
To further increase the implementation of eSET a new strategy is applied in Belgium. Since July 2003 the legislation prescribes the reimbursement of six cycles in a life-time per patient under the condition that in the first cycle of patients < 36 years, only one embryo is transferred, irrespective of the availability of a good quality embryo. In the subsequent cycles and in the cycles in older patients, transfer of a maximum of two or three embryos is allowed (Ombelet *et al.*, 2005). Studies comparing the IVF results before and after the implementation of the new legislation in Belgium showed no difference in the overall ongoing PR per cycle, and a reduction in the overall twin PR, although studying two different strategies in two subsequent time-periods has its limitations (Debrock *et al.*, 2005; Gordts *et al.*, 2005). Besides, in these studies only outcome per cycle in two time periods was compared instead of cumulative outcome per patient.
Our group published a study in which the transfer strategy was comparable to the transfer strategy in the first cycle in Belgium, although in our study no age limit was set (Van Montfoort *et al.*, 2006). In this randomized controlled trial (RCT) eSET was applied in the first cycle of an unselected patient population, i.e. irrespective of the availability of a good quality embryo and age. This strategy however led to a significant reduction in the ongoing PR as compared to DET or an eSET/DET policy in the first cycle (Van Montfoort *et al.*, 2006). Whether or not this decrease in PR and the reduction in twin PR have their influence on the total IVF outcome per patient after several cycles of an eSET/DET policy remains to be elucidated.

The aim of the present study was therefore to compare two treatment strategies, performed in the same time-period, which are similar for the second and third cycle (i.e. eSET/DET policy) but differ in the first cycle; one group receiving eSET irrespective of the availability of a good quality embryo and another group receiving an eSET/DET transfer policy. The two groups, consisting of patients < 38 years, were compared with respect to the total number of cycles, the live birth rate and the twin PR after a maximum of three cycles.

Methods

Patients
In the IVF clinic of the academic hospital Maastricht eSET is routinely performed in patients < 38 years when at least one good quality embryo is present (see below). If one of the criteria is not fulfilled, DET is performed. This transfer policy is referred to as the eSET/DET transfer policy and is applied in all treatment cycles of a patient. From January 2002 until December 2004, a RCT was performed in which consenting patients were randomly allocated to either eSET or DET in their first IVF or ICSI cycle, irrespective of the availability of a good quality embryo and female age. The only exclusion criteria were preimplantation genetic diagnosis (PGD), request of SET for medical reasons, language barrier (patients could not be well informed) and less than two 2PN embryos available. Patients fulfilling these criteria but declining to participate received our eSET/DET transfer policy (Van Montfoort *et al.*, 2006). This study was approved by the Institutional Ethical Board of the academic hospital of Maastricht.

In the present study the results of two transfer strategies were compared, which only differed in the first cycle: group A receiving eSET irrespective of the availability of a good quality embryo (i.e. patients randomized for eSET in our RCT) and group B receiving the eSET/DET transfer policy (i.e. patients declining to participate in our RCT). As the age limit for the eSET/DET policy in group B was 38 years, also in group A only patients < 38 years were included in the analysis. In the second and third cycle of all patients (a maximum of three cycles is reimbursed in the Netherlands) the eSET/DET transfer policy was applied. In case supernumerary embryos were cryopreserved, and no pregnancy was established, the cryopreserved embryos were transferred before a new ovum pick-up was performed. The transfer of these cryopreserved embryos was performed in all cycles of both groups according to the eSET/DET policy.

Ovarian stimulation, embryo culture and quality assessment
Ovarian stimulation, IVF, ICSI and embryo culture procedures have been described in detail earlier (Van Montfoort *et al.*, 2006). For each embryo originating from a normally fertilized oocyte, an embryo score was calculated on the basis of morphological grade (1 to 4, with grade 4 being the best grade), number of blastomeres and presence or absence of multinucleated blastomeres (MNBs) (Van Montfoort *et al.*, 2005). This score ranged on day 2 from 1-16.5 with a higher score correlating to a better embryo quality. Embryos that had reached the 4- or 5-cell stage on day two, or the 8-cell stage on day three, in combination with having the best morphological grade (regular, even sized blastomeres with less than 20% fragmentation) and an absence of MNBs were classified as good quality embryos (score ≥ 13.25 on day 2 or ≥ 23.25 on day 3) (Van Montfoort *et*

al., 2005). Embryos were transferred on day 2 after ovum pick-up or in a minority of cases, for reasons of convenience, on day 3. In all cases, including those in the RCT study, embryos with the highest embryo score were transferred.
Cryopreservation of supernumerary embryos was performed on the morning of the third day after ovum pick-up depending on their embryo score. The vast majority of cryopreserved embryos had reached the 7- to 8-cell stage in combination with morphological grade 3 or 4.

Statistical analysis
Each patient was followed until a live birth either after the transfer of fresh or frozen/thawed embryos was registered, the patient had completed an IVF or ICSI treatment with a maximum of three ovum pick-ups (and any frozen-thawed embryo transfers) without success or the patient dropped out for medical or personal reasons. In this study a cycle is defined as a stimulation cycle resulting in an ovum pick-up. The cycles not resulting in an ovum pick-up are left out of consideration. A miscarriage was defined as a proven loss of the pregnancy and/or no fetal heart beat on ultrasound examination at or before 12 weeks gestation in a patient with an initially positive hCG test at two weeks after embryo transfer (detection limit 50 IU/l in urine). An independent sample *t*-test was used to compare continuous variables, and the χ^2 test was used for binary variables. A *P*-value <0.05 was considered significant.

To analyse whether or not differences in drop-out rate between the two groups might interfere with the outcome (i.e. cumulative live birth rates) a life-table analysis was performed (Cooke *et al.*, 1981). As patients dropping out for medical reasons (e.g. poor response on ovarian stimulation) are assumed to have a lower live birth rate irrespective of the treatment strategy, these patients were excluded (Cooke *et al.*, 1981; Daya, 2005). Especially patients dropping out for personal reasons (e.g. relocation to other part of country or the burden of treatment) or because of a spontaneous pregnancy can influence cumulative outcome, as these patients are likely to have similar live birth rates as compared to patients not lost to follow up (Daya, 2005). As the live birth rate of the patients lost to follow up is unknown, life table analysis was performed with two different assumptions; the live birth rate in patients dropping out to be 0% and to be similar to that in patients remaining in the study.

Results

Group A consisted of 146 patients and group B of 175 patients. Those who had not completed their treatment at the time of analysis (23 months after the first ovum pick-up of the last patient included) were excluded from the analysis (5 and 1 patients respectively). Therefore the analysis was performed on 141 patients in group A and 174 patients in group B.
Both groups were comparable regarding patient and first cycle characteristics (Table I). Table II presents the clinical outcomes of the first cycle.. In group A all patients had eSET, and in group B eSET was performed in 100 patients (57.5%). The transfer of two embryos in 42.5% of the patients in group B led to a significantly higher live birth and twin PR as compared with group A (33.9% vs 21.3% and 13.3% vs 0%, respectively). After the transfer of cryopreserved embryos in patients not achieving a pregnancy in the fresh cycle, live birth and twin PRs were comparable (32.6% and 8.5% in group A and 39.7% and 14.3% in group B, Table II). In table III and IV the results of the first fresh cycles of group A and B are classified according to the transfer of good or moderate quality embryos. Although in group A in both cases one embryo was transferred, it did not result in a significant difference in clinical outcome (Table III). In group B one good quality embryo or two moderate quality embryos were transferred (Table IV). With the exception of the twin PR, no significant difference was found between eSET and DET in group B. Also no significant difference was found in clinical outcome between eSET with a good quality embryo in group A (Table III) and B (Table IV).

Table I : Patients' and first cycle characteristics

	Group A (n=141)	Group B (n=174)
Patients' characteristics		
Mean female age ± SE (year)	32.3 ± 0.3	32.8 ± 0.3
Duration of subfertility ± SE (year)	3.3 ± 0.1	3.4 ± 0.2
Primary subfertility (%)	107 (75.9)	115 (66.1)
Cause of subfertility (%)		
Tubal factor	22 (15.6)	27 (15.5)
Male factor	81 (57.4)	107 (61.5)
endometriosis	6 (4.3)	3 (1.7)
Unexplained	31 (22.0)	37 (21.3)
Other	1 (0.7)	
First cycle characteristics		
ICSI cycles (%)	83 (58.9)	105 (60.3)
IVF cycles (%)	58 (41.1)	69 (39.7)
Mean n°. of oocytes per retrieval ± SE	8.9 ± 0.4	9.0 ± 0.4
Fertilization rate ± SE	73.4 ± 1.6	73.6 ± 1.6
Rate of embryos with ≥ 4 blastomeres on day 2 per retrieval ± SE	72.8 ± 2.5	66.0 ± 2.3
Mean embryo score on day 2 ± SE	8.4 ± 0.2	8.3 ± 0.2
Mean no of transferred embryos ± SE	1.00 ± 0.0[a]	1.56 ± 0.5[b]
Mean no of frozen embryos per cycle ± SE	1.60 ± 0.2	1.29 ± 0.2
No. of cycles with ≥ 1 good quality embryo (%)	59 (40.4)	72 (41.4)

[a,b] Numbers with different superscripts within one row are significantly different (independent sample *t*-test for continuous variables and χ^2 test for binary variables, $P < 0.05$)

Table II: Clinical outcome of the first cycle for group A and B

	Group A, n=141 (%)	95% CI (%)	Group B, n=174 (%)	95% CI (%)
Fresh				
OPU	141		174	
eSET	141 (100)[a]	0 - 100	100 (57.5)[a]	50.2-64.8
Positive hCG test	46 (32.6)	24.9-40.3	75 (43.1)	35.7-50.5
Miscarriage	15 (32.6)	19.1-46.1	15 (20.0)	10.9-29.1
Pregnancy (12 wks)	31 (22.0)[a]	15.2-28.8	60 (34.5)[a]	27.4-41.6
Twin pregnancy	0 (0)[a]	-	8 (13.3)[a]	4.7-21.9
Live birth	30 (21.3)[a]	14.5-28.1	59 (33.9)[a]	26.9-40.9
Cycles with cryo-preservation	70 (49.6)	41.3-57.9	73 (42.0)	34.7-49.3
Cryopreserved				
Thaw cycles	59 0.4/ patient[a]	0.3-0.5	42 0.2/ patient[a]	0.2-0.3
Pregnancy (12 wks)	16 (27.1)	15.8-38.4	10 (24.4)	11.4-37.4
Twin pregnancy	4 (25.0)	3.8-46.2	2 (20.0)	0-44.8
Cumulative				
Pregnancy (12 wks)	47 (33.3)	25.5-41.1	70 (40.2)	32.9-47.5
Twin pregnancy	4 (8.5)	0.5-16.5	10 (14.3)	6.1-22.5
Live birth	46 (32.6)	24.9-40.3	69 (39.7)	32.4-47.0

[a] Numbers with similar superscripts within one row are significantly different (χ^2 test, $P<0.05$)

Table III: Clinical outcome of patients in group A (n=141) classified into receiving a good quality embryo or not in the first cycle

	Good quality embryo available n=55 (%)	95% CI (%)	Minor quality embryo available n=86 (%)	95% CI (%)
Positive hCG test	21 (38.2)	25.4-51.0	25 (29.1)	20.3-37.9
Miscarriage	7 (33.3)	13.1-53.5	8 (32.0)	13.7-50.3
Pregnancy (12 wks)	14 (25.5)	14.0-37.0	17 (19.8)	11.4-28.2
Live birth	14 (25.5)	14.0-37.0	16 (18.6)	10.4-26.8

Table IV: Clinical outcome of the first cycle of patients in group B receiving eSET or DET

	eSET, n=100 (%)	95% CI (%)	DET, n=74 (%)	95% CI (%)
Positive hCG test	45 (45.0)	35.2-54.8	30 (40.5)	29.3-51.7
Miscarriage	12 (26.7)	0.8-39.6	3 (10.0)	0-20.7
Pregnancy (12 wks)	33 (33.0)	23.8-42.2	27 (36.5)	25.5-47.5
Twin pregnancy	1 (3.0)[a]	0-8.8	7 (25.9)[a]	9.4-42.4
Live birth	33 (33.0)	23.8-42.2	26 (35.1)	24.2-46.0

[a] Numbers with similar superscripts within one row are significantly different (χ^2 test, $P<0.05$)

Table V: Clinical outcome of the total treatment for group A and B

	Group A, n=141 (%)	95% CI (%)	Group B, n=174 (%)	95% CI (%)
Fresh				
OPU	289 2.0/patient[a]	1.9-2.2	315 1.8/patient[a]	1.7-2.0
eSET	211 (73.0)[a]	67.9-78.1	136 (43.2)[a]	37.7-48.7
Positive hCG test	91 (64.5)	56.6-72.4	113 (64.9)	57.8-72.0
Miscarriage	28 (30.8)[a]	21.3-40.3	20 (17.7)[a]	10.7-24.7
Pregnancy (12 wks)	63 (44.7)	36.5-52.9	92 (52.9)	45.5-60.3
Twin pregnancy	5 (7.9)	1.2-14.6	12 (12.9)	6.1-19.7
Live birth	62 (44.0)	35.8-52.2	89 (51.1)	43.7-58.5
Cycles with cryopreservation	133 (46.0)	40.3-51.7	126 (40.0)	34.6-45.4
Cryopreserved				
Thaw cycles	112 0.8/patient[a]	0.7-0.9	85 0.5/patient[a]	0.4-0.6
Pregnancy (12 wks)	26 (23.2)	15.4-31.0	20 (23.5)	14.5-32.5
Twin pregnancy	4 (15.4)	1.5-29.3	3 (15.0)	0-30.6
Cumulative				
Pregnancy (12 wks)	89 (63.1)	55.1-71.1	112 (64.4)	57.3-71.5
Twin pregnancy	9 (10.1)	3.8-16.4	15 (13.4)	7.1-19.7
Live birth	88 (62.4)	54.4-70.4	109 (62.6)	55.4-69.8

[a] Numbers with similar superscripts within one row are significantly different (χ^2 test, $P<0.05$)

The outcomes of cycles two and three did not differ significantly between the two groups. Despite the significant difference in miscarriage rate after a maximum of three fresh cycles, the cumulative live birth and twin PR per patient were similar for both strategies (62.4% and 10.1% in group A and 62.6% and 13.4% in group B, Table V). Only the mean number of fresh and frozen cycles, patients performed before achieving a pregnancy or before having completed the maximum of three cycles without success, was significantly different (2.0 and 1.8 fresh cycles per patient and 0.8 and 0.5 frozen cycles per patient in group A and B respectively, Table V).

Most cryopreserved embryos in patients not achieving a live birth have been thawed during the study period, except for one embryo in group A and two in group B, all from patients who decided to refrain from transfer. In 29 patients achieving a live birth in the study period, embryos are still cryopreserved. Additionally, in two patients from both groups, cryopreserved embryos are still stored as these patients became pregnant spontaneously.

The drop-out rate after the first cycle was significantly different between the two groups. In group A, 3.2% (2.1% for medical and 1.1% for personal reasons) of the non-pregnant patients dropped out compared to 15.4% in group B (2.9% for medical and 9.6% for personal reasons and 2.9% because of achieving a spontaneous pregnancy). In group B there was no relation between the number of embryos transferred in the first cycle and the drop-out rate as 15.5% dropped out after eSET and 15.2% after DET. Between the second and third cycle these numbers were 17.5% (4.8% for medical and 9.5% for personal reasons and 3.2% because of spontaneous pregnancies) for group A and 13.6% (1.7% for medical and 11.9% for personal reasons) for group B. To analyse whether this difference in drop out rate can influence cumulative outcome data, a life-table analysis was performed. Assuming a live birth rate in patients dropping out of 0% resulted in a cumulative live birth rate of 64.7% in group A and 64.1% in group B. When assuming a similar live birth rate in patients dropping out and those remaining in the study, these data were 66.5% and 68.6% respectively (both differences not significant).

Discussion

From the results of this study it became apparent that treatment strategies consisting of eSET (group A) or eSET/DET (group B) in the first cycle and eSET/DET in the second and third cycles in patients < 38 years led to similar live birth rates. To obtain this result, more cycles per patient were required in group A, while the overall twin PR was not significantly reduced in group A. Our results put the policy with eSET in the first cycle, irrespective of the availability of a good quality embryo, at a relative disadvantage, which is remarkable considering that for example in Belgium the legislation has been changed towards an eSET 'irrespective of the availability of a good quality embryo' policy in patients < 36 years (Gordts *et al.*, 2005). Although the overall cumulative live birth rate was similar in our study, the live birth rate in the first cycle was significantly different. This difference might be explained by the fact that approximately half of all human preimplantation embryos may be considered chromosomally abnormal (Coonen *et al.*, 1994; Baart *et al.*, 2006).

These aneuploidies do not affect the lower quality embryos only, but also morphologically normal embryos (Baart *et al.*, 2006; Munne *et al.*, 2006). In the case of eSET, approximately half of the embryos will be chromosomally abnormal and therefore not result in a pregnancy, even when this embryo is of high morphological quality. This can also be seen in Table III, where the transfer of one good or one moderate quality embryo was shown to lead to statistically similar live birth rates. After the transfer of two embryos in DET, there is a higher chance that at least one chromosomally normal embryo will be replaced, increasing the chance for pregnancy and live birth. It may be argued, however, that in eSET there are also two embryos available for transfer (one is transferred as a fresh embryo and the other after freezing and thawing), with a similar chance for at least one chromosomally normal embryo to be transferred as compared to the transfer of two fresh embryos in DET. In the eSET policy, however, part of the cryopreserved embryos will not survive the thawing process and will be lost before transfer. Therefore, although group A and B started with the same number of embryos (6.2 and 6.0 respectively), the net number of embryos available for transfer and therefore the net number of euploid embryos, will be lower in an eSET policy (group A) as compared to an eSET/DET policy (group B). This difference although not significant, did result in one extra cycle per five patients after eSET in our study.

In addition, the overall twin PR could not be reduced with this strategy. The transfer of cryopreserved embryos and the second and third fresh cycle were all performed according to an eSET/DET policy. The resulting twins annihilated the reduction in twin pregnancies after the first fresh cycle. As 44% of the twins in group A and 20% of the twins in group B originated

from the transfer of two frozen/thawed embryos, more attention should be paid to single embryo transfer with these embryos. Furthermore, more studies are needed to define the morphological criteria of cryopreserved embryos suitable for eSET.

Although the miscarriage rate between the two groups, defined as number of pregnancy losses ≤ 12 weeks of gestation per positive hCG test, did not differ significantly per cycle, there was a trend towards a higher miscarriage rate in group A as compared with group B (respectively 32.6% (15/46) vs 20.0% (15/75) for the first cycle, 24.2% (8/33) vs 11.1% (3/27) for the second cycle and 45.5% (5/11) and 18.2% (2/11) for the third cycle). Especially in the second and third cycle, the numbers were very small, but they nevertheless resulted in a significantly different cumulative miscarriage rate. The difference in the first cycle miscarriage rate, although not significant, was also reported in RCTs on eSET vs DET (41% vs 9% (Gerris *et al.*, 1999) and 30% vs 20% per clinical pregnancy (Lukassen *et al.*, 2005). However, other RCTs reported lower miscarriage rates for eSET compared to DET (15% vs 16% (Thurin *et al.*, 2004) and 4% vs 9% (Martikainen *et al.*, 2001)). The reason for the difference in miscarriage rate in our study between group A and B remains elusive. As for group A the miscarriage rate is similar for good and moderate quality embryos, embryo quality cannot be regarded as the cause for the difference in miscarriage rate. It can, however, partly be explained by the fact that only positive hCG tests resulting in a complete loss of the implantation were counted as a miscarriage. The loss of one conceptus in case of DET (vanishing twin) before or after the detection of a fetal heart beat is not included and the chance of losing both embryos in case of DET is smaller as compared to the loss of one embryo in eSET, putting the latter at a disadvantage.

The drop-out rate in the first cycle of group B was significantly higher as compared to A (15.4% vs 3.0%). The most prominent cause for drop-out in group B were personal reasons, while almost no couple dropped out in group A for this reason. As in group B the drop-out rate was similar after eSET and DET, the number of embryos transferred cannot explain this difference. A more likely explanation is that the first cycle of group A was in a study setting where patients not obtaining a pregnancy in the first three cycles were offered a fourth cycle free of charge, while in group B it was part of standard care. The drop-out rate in group B (15.4%) is comparable to the one reported for standard care IVF (26.2%) (Land *et al.*, 1997). Patients agreeing to participate in the RCT study are possibly more motivated to continue treatment and less likely to drop out. Life-table analysis, using both a minimal and a maximal assumed live birth rate in the drop-out group led to comparable cumulative live birth rates in groups A and B.

From this it can be concluded that the difference in drop-out rate did not influence the comparison of the two transfer strategies.

From this study we conclude that in order to reduce twin PRs, performing eSET irrespective of the availability of a good quality embryo, in all patients <38 years, in the first cycle only is not effective.

Early cleavage is a valuable addition to existing embryo selection parameters: a study using single embryo transfers

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Abstract

BACKGROUND: To reduce the twin pregnancy rate (PR), elective single embryo transfer (eSET) is increasingly implemented. Improvement of the results obtained with eSET can be achieved by better selection of the most viable embryo. This study investigated the predictive value of early cleavage (EC) as an additional parameter for selecting the embryo with the highest implantation potential by using data from SETs.

METHODS: Data from 165 SETs were retrospectively evaluated. Cleavage to the 2-cell stage was determined 23-26h after injection or 25-28h after insemination. Selection of the embryo to be transferred was based on cell morphology and cell number on the day of transfer, not on the EC status. Additional information on the predictive value of EC on developmental potential was obtained by analyzing 253 transfers with two embryos (double embryo transfer, DET) and blastocyst formation of 1160 surplus embryos. Logistic regression was used to determine the predictors of pregnancy or blastocyst development.

RESULTS: A significantly higher PR was observed after transfer of single EC embryos compared to single non-EC embryos (46 versus 18%). This result was confirmed by the significantly higher PR after DET with two EC embryos as compared to DET with two non-EC embryos (45 versus 25%) and the blastocyst formation of EC embryos compared to non-EC embryos (66 versus 40%). Logistic regression showed that EC is an independent predictor for both pregnancy and blastocyst development in addition to cell morphology and cell number.

CONCLUSIONS: In order to improve the selection of the embryo with the highest implantation potential, selection for transfer should not be based on cell number and morphology on the day of transfer alone, but also on EC status.

Introduction

A twin pregnancy is an adverse outcome of an IVF cycle, because of the increased risk for medical complications including prenatal and neonatal mortality and morbidity, preterm delivery, low birth weight and long-term medical and developmental problems (Elster, 2000; ESHRE Capri Workshop, 2000; Olivennes, 2000; Land and Evers, 2003). As a result, the medical costs of a twin pregnancy increase dramatically compared to the costs for a singleton pregnancy (Elster, 2000; Kinzler *et al.*, 2000). In order to prevent this adverse outcome, many recent studies focus on elective single embryo transfer (eSET). The results show that eSET in a selected group of patients leads to an acceptable pregnancy rate (PR) and a reduction in the twin PR (Gerris *et al.*, 1999; Vilska *et al.*, 1999; Martikainen *et al.*, 2001; Gerris *et al.*, 2002).

As eSET is increasingly performed, there is an urgent need for better criteria to select the embryo with the highest implantation potential. In some IVF centres, embryos are cultured until blastocyst stage to make a better selection. However, in several studies, blastocyst transfer has not been demonstrated to be a better alternative for transfer of cleavage stage embryos (Rienzi *et al.*, 2002) and many centres, including our own, still prefer the transfer of the cleavage stage embryo. At present, the common practice of selecting the most viable cleavage stage embryo is based on embryo morphology and developmental stage on the day of transfer (Giorgetti *et al.*, 1995; Ziebe *et al.*, 1997; Van Royen *et al.*, 1999). Embryo morphology is determined by the number, size and shape of blastomeres, the proportion of fragments and in some studies the presence of multinucleated blastomeres (MNB). It has been demonstrated that after 2 days of culture, the 4- or 5-cell stage is the optimal cleavage stage (Ziebe *et al.*, 1997; Van Royen *et al.*, 1999). Embryos at this cleavage stage with little or no fragmentation and an absence of MNBs achieved a higher implantation and PR compared to embryos at another cleavage stage with more fragmentation (Ziebe *et al.*, 1997; Van Royen *et al.*, 1999).

Recently, new parameters to improve embryo selection have been introduced, e.g. zygote morphology and early cleavage (EC). The morphological parameters for zygote quality include the number of nucleolar precursor bodies (NPB) and their distribution in the pronuclei (Scott *et al.*, 2000; Tesarik *et al.*, 2000; Wittemer *et al.*, 2000). EC embryos were defined as those which had cleaved to the 2-cell stage 25-27h after insemination or ICSI (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Bos-Mikich *et al.*, 2001; Lundin *et al.*, 2001; Sakkas *et al.*, 2001; Fenwick *et al.*, 2002; Tsai *et al.*, 2002). The conclusion of all these studies was that transfer of EC embryos leads to a higher pregnancy and implantation rate compared to the transfer of non-EC embryos. However, most studies examined transfers of more than one embryo, where only one embryo had to be an EC embryo. As a result, many cycles were included where both early and non-

EC embryos were transferred. Therefore it remains uncertain whether the pregnancy or implantation can be attributed to the EC embryo (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Bos-Mikich *et al.*, 2001; Sakkas *et al.*, 2001; Fenwick *et al.*, 2002; Tsai *et al.*, 2002).
Furthermore, it is still unclear whether EC is an independent predictor for pregnancy or whether it is correlated with other pregnancy predictors e.g. embryo morphology and cell number on the day of transfer. In several studies it has been shown that EC embryos had a significantly higher cell number and a better embryo morphology on the day of transfer, compared to non-EC embryos (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Lundin *et al.*, 2001; Sakkas *et al.*, 2001; Fenwick *et al.*, 2002). The aim of the present study was to assess the predictive value of EC for pregnancy in relation to embryo quality, i.e. embryo score. Only SET data were analysed. Surplus embryos were used to evaluate embryonic development to the blastocyst stage. Logistic regression was performed to assess the predictive value of EC for both pregnancy and blastocyst development.

Materials and methods

Patients
During a 2 year period (May 2001 to June 2003), all IVF and IVF/ICSI cycles performed in the academic hospital Maastricht that fulfilled the following criteria were included in the present study. First, EC of embryos had to be determined between 23 and 26h post-injection or 25 and 28h post-insemination, the time difference being necessary to compensate for the time difference in early development between IVF- and ICSI-derived embryos (Nagy *et al.*, 1994; Nagy *et al.*, 1998). Second, there had to be at least one normally fertilized (2PN) zygote present. Third, the indication for IVF treatment should not be preimplantation genetic diagnosis (PGD).
During this period, either one or two embryos were transferred. To evaluate the predictive value of EC for pregnancy, data collected from a total of 165 cycles in which only a single embryo was transferred on the second day after oocyte retrieval were analysed. SET was performed when: a) only one embryo was available (n = 30); b) a top quality embryo (4-cell, grade 4 and no MNBs) was available, and the woman was aged < 38 years of age, which leads according to our standard transfer policy to eSET (n = 77); c) patients agreed to participate in a research project in which one or two embryos were transferred at random irrespective of embryo score (n = 47); d) there were clear medical or socio/psychological reasons for eSET, e.g. uterus anomaly (n =11).
Besides the 165 SET procedures, a total of 253 double embryo transfer (DET) procedures were analyzed, in which either two EC embryos or two non-EC embryos were transferred.
In addition, 1160 surplus embryos originating from 341 cycles remaining

after transfer and not suitable for freezing because of inferior quality were cultured until day 6 for analyzing the blastocyst formation rate, provided that the patients had signed an informed consent. This study was approved by the local Ethics Commitee.

Ovarian stimulation protocol
Patients were down-regulated with triptorelin daily s.c. injections (Decapeptyl; Ferring B.V. The Netherlands) or nafarelin intranasally (Synarel; Searle BV, The Netherlands). To stimulate multiple follicular development, recombinant FSH (Puregon, Organon, The Netherlands) was used. Follicle growth was monitored by ultrasound. As soon as at least three follicles were ≥ 18 mm 5000 IU of hCG (Pregnyl, Organon) was given. Ultrasound-guided oocyte retrieval was performed 36 h after hCG administration.

IVF and ICSI procedure

IVF and ICSI procedures used were as described in detail earlier (Dumoulin *et al.*, 2000; Dumoulin *et al.*, 2001). Oocytes and embryos were cultured individually in 5 µl droplets covered by mineral oil in an atmosphere of 5% O_2, 5% CO_2 and 90% N_2. Culture medium G1.2 (Vitrolife, Sweden) was alternated with Sydney IVF Cleavage media (Cook, Australia) for each consecutive treatment cycle.

Embryo quality assessment and transfer policy
For each embryo originating from a normally fertilized (2PN) oocyte, an embryo score was calculated on day 2 by multiplying the morphological grade (1 to 4 grading system of Bolton *et al.* (1989) in which grade 4 is morphologically the best) with the number of blastomeres (Steer *et al.*, 1992). As it has been shown that the 4- or 5-cell stage is the optimal cleavage stage at day 2 and that the presence of MNB is deleterious for embryo viability (Ziebe *et al.*, 1997; Van Royen *et al.*, 1999; Van Royen *et al.*, 2003), the score for these embryos was adjusted as follows: embryos consisting of >5 cells were multiplied by 0.9, embryos showing MNB as a 2-cell embryo on any of the two observation time-points (23-26h post-injection or 25-28h post-insemination and 41-45h post-injection/insemination) were multiplied by 0.5 and embryos showing MNB as a > 2-cell embryo were multiplied by 0.75. The calculation of embryo scores was performed to make a uniform selection of the embryo to be transferred. In our embryo score system, a 4-cell embryo with morphology grade 4 and an absence of MNB receives the highest score of 16.
Embryos with the highest score available on day 2 were transferred irrespective of the EC status.
Cryopreservation of supernumerary embryos was performed on the morning of the third day if one or more embryos had reached the 8-cell stage, and if they were of good morphological quality. Embryos left over after transfer and not suitable for freezing from consenting patients were

transferred alternately to droplets of G2.2 medium or Sydney IVF Blastocyst medium and cultured until the sixth day after ovum retrieval. Each day developmental stages were recorded.
Pregnancy was determined by a urine hCG test 14 days post transfer. An ongoing pregnancy was defined as the presence of at least one intrauterine gestational sac with fetal cardiac activity at ultrasound examination, 5-6 weeks after ovum retrieval.

Statistical analysis
Statistical analysis was performed using the SPSS software for Windows version 10.1 (Statistical Package for Social Sciences, Inc., USA). To compare the differences between the EC embryos and non-EC embryos, Student's *t*-test was used for the continuous variables and the χ^2 test was used for binary variables. To evaluate the impact of embryo score and EC on pregnancy and blastocyst development, two models were constructed by logistic regression analysis. Embryo score was a continuous variable ranging from 1 to 16. EC was a binary variable (EC 1, non-EC 0). To take into account the possible interaction between embryo score and EC status, an interaction factor between those two variables was also included as a possible independent variable in the logistic regression. By using the equation derived from the logistic regression analysis, a predicted probability for both pregnancy and blastocyst development can be calculated for every embryo score. Based on this predicted probability, a receiver operating curve (ROC), plotting the true positives against the false positives, was constructed. The area under the receiver operating curve (AUC_{ROC}) was used to summarize the predictive power of the model, ranging from 0 to 1. A receiver operating curve (ROC) plots the true positives against the false positives. $P < 0.05$ was considered significant.

Results

From the 1134 ovum retrievals performed in the study period, 418 were included in the present study (165 SET and 253 DET). To investigate the predictive value of EC as an additional parameter for selecting the embryo with the highest implantation potential, the data from the 165 SETs were used. In 71 of these cycles, standard IVF was performed, while in the remaining 94 cycles, ICSI was performed. The proportion of cycles in which ICSI was applied (58%) was comparable to that of the total IVF-programme during the study period (54%). Of all embryos obtained, 39% of the ICSI embryos and 38% of the IVF embryos were early cleavers. Of the 165 embryos that were transferred, 97 (59%) were EC embryos, while 68 (41%) were non-EC embryos. The results including embryo score and PR are shown in Table I. Both embryo score and PR differ significantly between EC and non-EC embryos.

To examine the relationship between EC, embryo score and pregnancy, a logistic regression was performed. EC was a significant predictor when adjusted for embryo score (odds ratio (OR) = 3.24; $P < 0.05$). The interaction between embryo score and EC status was not significant. In Figure 1 the predicted PRs when transferring an EC embryo or a non-EC embryo are plotted for each embryo score. The ratio between the probability for pregnancy of EC and non-EC embryos decreases with increasing embryo score. The predictive power of this pregnancy model calculated by the AUC_{ROC} was 0.68.

To confirm the positive impact of EC on PR, 253 DETs were analysed (Table II). In 97 cases where two EC embryos were transferred, the PR was significantly higher compared to the 156 transfers where two non-EC embryos were transferred (45 versus 25%). In addition, a total of 1160 surplus embryos from 341 cycles, which were examined for EC as well, were cultured until day 6 after ovum pick-up. Table III shows the blastocyst formation rate and embryo score for both groups. Twenty-three percent of the oocytes which were injected cleaved early, compared to 24% of the inseminated oocytes. The logistic regression analysis for blastocyst development showed that embryo score ($P < 0.0001$) and EC ($P < 0.0001$) were significant predictors. In addition, there was also a significant interaction between these two predictors ($P < 0.05$), which means that the effect of EC on blastocyst development is dependent on the embryo score. The advantage of EC for blastocyst development decreases as the embryo score increases. This can also be seen in Figure 2 where the predicted probabilities for blastocyst development based on the logistic regression model are shown. The ratio between the probability for blastocyst development of EC and non-EC embryos decreases as the embryo score increases. Because of this extra independent variable, the OR of EC also becomes dependent on embryo score (Figure 3). The OR decreases as the embryo score increases. The AUC_{ROC} as a measure for the predictive value of this model is 0.72.

Table I: Cycle characteristics of single embryo transfers with an early cleavage embryo and single embryo transfers with a non-early cleavage embryo

	Early eavage	No early cleavage	*P* value
No. of SET cycles	97	68	
No. of ICSI embryos (%)	203 (39)	313 (61)	
No. of IVF embryos (%)	161 (38)	268 (62)	
Female age (mean ± SD)	32.9 ± 2.9	32.9 ± 3.4	0.7
No. of oocytes (mean ± SD)	10.7 ± 0.7	8.9 ± 0.8	0.09
Day 2 embryo score (mean ± SD)	13.4 ± 2.1	10.0 ± 4.5	*P*<0.0001
Pregnancy rate (%)	45/97 (46.4)	12/68 (17.6)	*P*<0.001
Ongoing pregnancy rate (%)	36/97 (37.1)	7/68 (10.3)	*P*<0.001
Abortion rate (%)	9/45 (20)	5/12 (41.7)	0.12

Table II: Cycle characteristics of double embryo transfers with two early cleavage embryos and double embryo transfers with two non-early cleavage embryos

	Early cleavage	No early cleavage	*P* value
No. of DET cycles	97	156	
Pregnancy rate (%)	44/97 (45)	39/156 (25)	*P*< 0.001

Table III: Comparison of characteristics from early cleavage and non-early cleavage surplus embryos

	Early cleavage	No early cleavage	*P* value
No. of embryos	274	886	
No. of ICSI embryos (%)	145 (23)	476 (77)	
No. of IVF embryos (%)	129 (24)	410 (76)	
Day 2 embryo score (mean ± SD)	7.0 ± 3.0	5.5 ± 2.9	*P*<0.0001
Blastocyst rate (%)	181/274 (66.1)	352/886 (39.7)	*P*<0.0001

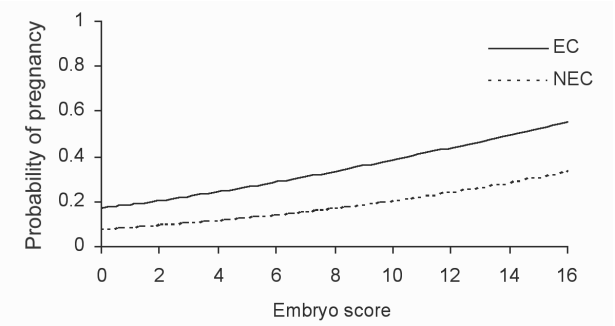

Figure 1: Predicted pregnancy rates for early cleavage (EC) and non-earl cleavage (NEC) embryos derived from a logistic regression model

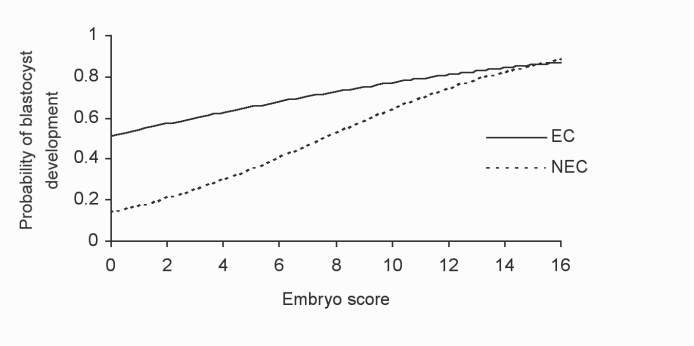

Figure 2: Predicted probability for blastocyst development for early cleavage (EC) and non-early cleavage (NEC) embryos from logisticregression models

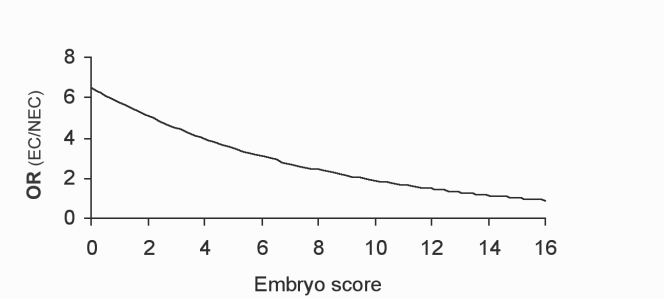

Figure 3: OR for blastocyst development between early cleavage and non-early cleavage embryos in relation to embryo score

Discussion

In order to select the most viable embryo, embryo scoring systems have been developed based on embryo morphology and blastomere number on the day of transfer (Cummins *et al.*, 1986; Steer *et al.*, 1992; Giorgetti *et al.*, 1995; Ziebe *et al.*, 1997). Much effort has been devoted to refining existing embryo scoring systems and finding additional simple, non-invasive parameters that could improve the embryo selection procedure (Van Royen *et al.*, 1999; Tesarik *et al.*, 2000; Wittemer *et al.*, 2000). EC status is one of the most promising new parameters.

In previous studies it has been found that transferring embryos that had cleaved early led to a significantly higher PR (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Bos-Mikich *et al.*, 2001; Lundin *et al.*, 2001; Sakkas *et al.*, 2001; Fenwick *et al.*, 2002; Tsai *et al.*, 2002) and blastocyst development rate (Fenwick *et al.*, 2002) as compared to non-EC embryos. This is in accordance with the results obtained in the present study (46 versus 18% for PR after SET, $P < 0.001$, and 66 versus 40% for blastocyst rate, $P < 0.0001$). However, most studies examined transfers in which two or more embryos were transferred of which at least one embryo had shown EC. This makes it difficult to conclude to which embryo the pregnancy can be attributed. In our study, SET data were analyzed, which makes it possible to determine the relationship between EC and pregnancy arising from one specific embryo. Recently, Salumets *et al.* (2003) also analyzed SET data; their results for PRs are similar to ours (50 versus 26.4%, $P = 0.001$). Furthermore, our results after DET with either two EC embryos or two non-EC embryos (PR 45 versus 25%, $P < 0.001$), confirm the findings in the SET group. EC is now used in a few clinics as a selection criterion, but only to make a distinction when more embryos of identical embryo score are available for transfer (Sakkas *et al.*, 1998; Salumets *et al.*, 2003).

Concerning the relation between EC status and embryo quality, it was found previously that, as our results show as well, the early-cleaving embryos had a significantly higher embryo score on the day of transfer (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Lundin *et al.*, 2001; Fenwick *et al.*, 2002). Sakkas *et al.* (1998) already suggested that this might indicate that an EC embryo would have been selected for transfer anyway, based on cell stage and morphology. This would imply that EC status analysis might be redundant. However, as the results from our logistic regression analysis indicate, both embryo score, calculated before embryo transfer, as described in materials and methods, and EC status are independent predictors for both pregnancy and blastocyst development. In the blastocyst model, an interaction exists between embryo score and EC, which is not significant in the pregnancy model. This discrepancy can be explained by the fact that the blastocyst data were more numerous than the pregnancy data.

Our findings imply that EC status should always be taken into account when selecting the best embryo for transfer and not only when more embryos of identical embryo score are available for transfer. This is not in accordance with the findings of Lundin *et al.* (2001) who found that, after a logistic regression analysis, EC status was not an independent predictor of pregnancy. However, when the analysis was performed separately for ICSI and IVF, EC was shown to be an independent predictor for birth in ICSI cycles, but not in IVF cycles. They also found that embryos derived after ICSI cleaved earlier compared to those after IVF (Lundin *et al.*, 2001). This has been explained by Nagy *et al.* (1998). By injecting the spermatozoon into the oocyte, the zona pellucida and cumulus and corona cells barrier is overcome. This gives the ICSI embryos a temporal advantage of 4h, which might explain why EC was an independent predictor for birth after ICSI, but not after IVF. According to our data this time span was only 2h (data not published). That is the reason why the time interval of 2h was used between the EC status determination of IVF and ICSI embryos.
The predictive value of the pregnancy model is relatively weak (AUC=0.68). This is likely due to the fact that there are many other oocyte and embryonic factors which might influence the implantation capacity of the embryo. In addition, a pregnancy is not only dependent on embryo quality, but also on endometrial receptivity, for example.

In our study, it was found that the abortion rate was increased in the non-EC group compared to the EC group (42 versus 20%). Although the difference is not statistically significant, it is in accordance with the findings of Lundin *et al.* (2001) who were able to analyse a greater number of cycles.
As in the present study we find that EC has a predictive value both for implantation after transfer of cleavage stage embryos, as well as for blastocyst formation of surplus embryos, it might be expected that EC also has a predictive value for implantation after transfer of blastocysts. This is, however, not examined in the present study.

In conclusion, this study provided data from a series of SETs showing that EC is a significant predictor of both pregnancy and blastocyst development. Therefore, in order to improve the selection of the embryo with the highest implantation potential, selection of embryos for transfer should not be based on cell number and morphology on the day of transfer alone, but also on EC status. EC stage analysis provides means of improvement of the embryo selection process that might lead to a transfer policy with a higher proportion of SETs. In turn, this will lead to a reduction of the twin PR, which is one of the most serious adverse outcomes of an IVF treatment for both mother and child.

Differential gene expression in cumulus cells as a prognostic indicator of embryo viability: a microarray analysis

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Abstract

Besides the established selection criteria based on embryo morphology and blastomere number, new parameters for embryo viability are needed to improve the clinical outcome of in vitro fertilization (IVF) and more particular of elective single embryo transfer (eSET). Genome-wide gene expression in cumulus cells was studied, since these cells surround the oocyte inside the follicle and therefore possibly reflect oocyte developmental potential. Early cleavage (EC) was chosen as a parameter for embryo viability. Gene expression in cumulus cells from eight oocytes resulting in an EC embryo (EC-CC; n=8) and from eight oocytes resulting in a NEC embryo (NEC-CC; n=8) was analysed using microarrays (n=16). The resulting embryos had all reached the 4-cell stage at day 2 with a similar morphology grade. Between the EC-CC and NEC-CC samples, 611 genes were differentially expressed ($P < 0.01$). These genes are mainly involved in cell cycle, angiogenesis, apoptosis, EGF, FGF and PDGF signaling, general vesicle transport and chemokine and cytokine signaling. To validate the microarray results quantitative real-time PCR (qRT-PCR) of 25 selected genes was performed on the original microarray samples as well as on 24 (12 EC-CC and 12 NEC-CC) extra independent samples Fifteen (60%) genes analysed could be validated in the original samples and of these 8 (53%) could also be validated in independent samples. The most differentially expressed genes among these were CCND2 (cell cycle), CXCR4 (chemokine signaling), GPX3 (peroxidase), CTNND1 (cell adhesion), DHCR7 (steroid metabolism), DVL3 (Wnt signaling), HSPB1 (stress response) and TRIM28 (chromatin remodeling). This study shows that the differential expression of several genes in cumulus cells can be related to embryo development. This opens up perspectives for a new molecular embryo or oocyte selection parameter which might also be useful in countries where the selection has to be made at the oocyte stage before fertilization instead of at the embryonic stage.

Introduction

The only way to prevent a dizygotic twin pregnancy in IVF, which is regarded as one of the most serious complications, is single embryo transfer (SET). As most patients have more than one embryo available for transfer, selecting the most viable one is of pivotal importance. Most clinics rely for embryo selection on the non-invasive examination of developmental and morphological aspects of the embryos. In every stage of oocyte and embryonic development, characteristics have been defined which appear to be prognostic indicators of successful pregnancy. Among these are zona pellucida thickness and cytoplasmic granularity of the oocyte, size of pronuclei and alignment of nuclear polar bodies in the zygote, early cleavage in the cleavage stage embryo and number and size of blastomeres, fragmentation and multinucleation in the 4-8 cell stage embryo (see Borini *et al.* (2005) for a review and Gerris (2005) for a more extensive list of references). Especially early cleavage (EC) appears to be a good parameter for embryo viability as it is highly correlated with the blastocyst formation rate (Fenwick *et al.*, 2002) and the implantation and pregnancy rate (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Lundin *et al.*, 2001), not only in double but also in single embryo transfers (Salumets *et al.*, 2003; Van Montfoort *et al.*, 2004). For embryos in the intermediate syngamy state, the pregnancy rate was in between that of EC and non-early cleavage (NEC) embryos (Wharf *et al.*, 2004).

As the developmental potential of an embryo cannot be fully determined by characteristics visible by microscopy alone, several other markers are studied (Pearson, 2006). For instance, several investigators focussed on the influence of the follicular micro-environment on subsequent embryonic development. The follicular fluid LH and growth hormone levels at the time of oocyte retrieval were higher in embryos with good morphology (Mendoza *et al.*, 2002). Furthermore, the concentrations of hormones (17β-estradiol, LH, growth hormone, prolactin, leptin), growth factors (insulin-like growth factor-I), cytokines (interleukin-1) and proteinases (matrix-metalloproteinase-9) in follicular fluid differ according to the probability of pregnancy (Mendoza *et al.*, 2002; Anifandis *et al.*, 2005; Hammadeh *et al.*, 2005; Lee *et al.*, 2005). Also vascularization of the follicles has been examined as a potential marker for the developmental potential of an embryo. The peri-follicular blood flow characteristics are related to oocyte oxygenation (Van Blerkom *et al.*, 1997) and can differ between the follicles in one ovary. Nargund *et al.* (1996) found, by Doppler imaging of the follicular blood flow, that oocytes from poorly vascularized follicles developed in morphologically inferior embryos as compared to those from well vascularized follicles. Several other studies confirmed a positive relationship between perifollicular vascularization and pregnancy (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997; Borini *et al.*, 2004). Pregnancies were only achieved with embryos from oocytes which

had vascularity detected in > 50% of their follicular circumference and live births only from oocytes with > 75% follicular vascularity (Chui *et al.*, 1997). Gaulden *et al.* (1992) suggested that hypoxic intracellular conditions might result in a diminished level of oxidative metabolism in the oocyte and a lower intracellular pH. The latter in turn could lead to meiotic spindle instabilities and chromosomal abnormalities. Indeed, Chiu *et al.* (1997) and Van Blerkom *et al.* (1997) reported a significantly higher incidence of aneuploidy and spindle defects in oocytes derived from follicles with poor vascularization as compared to those from well-vascularized follicles. In addition, ATP content of the oocyte and dissolved oxygen content of the follicle fluid are related to oocyte/embryo development (Van Blerkom *et al.*, 1995; Van Blerkom *et al.*, 1997).

As the oocyte is in dialogue with the surrounding cumulus cells via paracrine and gap-junctional signaling (Sutton *et al.*, 2003), we hypothesized that differences in intra-follicular processes which are responsible for oocyte and embryonic development and subsequently implantation are reflected in the gene expression pattern of cumulus cells. The bi-directional communication between the oocyte and the cumulus cells is necessary for oocyte development as oocytes fail to grow in the absence of (a connection with) cumulus cells (Ackert *et al.*, 2001). Zhang *et al.* (2005) reported that the expression of several genes in cumulus cells, particularly pentraxin 3, was indicative of oocyte and embryo quality. In turn, oocyte factors like growth and differentiation factor-9 (GDF-9) are necessary for cumulus expansion (Sutton *et al.*, 2003).

The aim of this study was to analyse the genome-wide expression of genes in cumulus cells as indicators of embryo viability. By analysing gene expression in cumulus cells, the understanding of the regulation of oogenesis and embryonic development might be improved. This information might lead to new molecular non-invasive embryo selection parameters reflected in cumulus cells that can be used in addition to the existing morphological parameters or might result in an oocyte selection tool for those who are obliged to select a limited number of oocytes for fertilization (Ludwig *et al.*, 2000).

Methods

Patients and human cumulus cell collection

Patients visiting the IVF clinic of the academic hospital Maastricht underwent an IVF or ICSI treatment as described previously (Van Montfoort *et al.*, 2006). For the study, which was approved by the local Ethics Committee, in consenting patients, immediately following ultrasound-guided cumulus-oocyte-complex (COC) retrieval, a proportion of the cumulus cells surrounding a single oocyte were removed using a sharp needle, lysed in 100 µl Trizol reagent (Invitrogen, Carlsbad, USA) supplemented with 1% (v/

v) 2-mercapto-ethanol (Merck, Darmstadt, Germany), snap-frozen in liquid nitrogen and stored at -80°C (cumulus cells from one oocyte per vial). The oocytes were cultured and fertilized individually in 5 µl droplets covered by mineral oil. Between 23-26 h post-injection or 25-28 h post-insemination early cleavage (EC) status of embryos was assessed. A 2h time difference is necessary to compensate for the time difference in early development between IVF- and ICSI-derived embryos (Van Montfoort *et al.*, 2004). Subsequently, on day two of development, the embryos were examined for morphology, number of blastomeres and the presence or absence of multinucleated blastomeres (MNBs) (Van Montfoort *et al.*, 2005).

Experimental design
EC was chosen as a marker for embryo viability. Gene expression in cumulus cells from eight oocytes resulting in an EC embryo (EC-CC; n=8) and from eight oocytes resulting in a non-EC embryo (NEC-CC; n=8) derived from six patients were analysed using microarrays (n=16). To exclude a differential gene expression due to differences in patient characteristics, samples were paired. From four patients both an EC-CC and a NEC-CC sample were used. From two additional patients two EC-CC as well as two NEC-CC samples were used. The microarray results were validated by quantitative real-time polymerase chain reaction (qRT-PCR) on the original samples analysed by microarray as well as on 24 'new' samples.
The cumulus cell samples (for microarray and RT-PCR) from EC and NEC embryos, were derived from normally fertilized (2PN) oocytes, which developed into embryos with comparable characteristics on day 2, i.e. 4-cell with good morphology and no MNBs present.

RNA isolation
Total RNA was extracted using Trizol reagent (Invitrogen, Carlsbad, USA) according to the manufacturers' instructions with some adaptations for the small quantity of RNA. RNA was precipitated with isopropyl alcohol for 2h and the RNA pellet was washed three times with 75% ethanol. To be able to track the small RNA pellet, 5 µg glycogen (Ambion, Woodward, USA) was added to the sample before RNA precipitation. Total RNA was resuspended in 20 µl RNAse free water and stored at -80°C. For all RNA samples quantity and purity were determined using the Nanodrop ND-1000 spectrophotometer (Nanodrop Technologies, Wilmington, USA) and RNA integrity was determined using the Bioanalyzer 2100 (Agilent Technologies, Palo Alto, USA).

Two cycle amplification and microarray hybridization
Fifty ηg total RNA was amplified using the two-cycle cDNA synthesis kit (Affymetrix, Santa Clara, USA) in combination with the MEGAscript T7 in vitro transcription system (Ambion). Biotin labelled target complementary RNA (cRNA) was fractionated and hybridized to Human Genome U133A

Plus 2.0 Arrays (Affymetrix). Each array contained more than 54,000 oligonucleotide probe-sets corresponding to 38,500 characterized human genes.

Microarray analysis

To identify probe sets which were differentially expressed between 8 EC-CC and 8 NEC-CC samples, a three step process was applied. Firstly, Affymetrix GeneChip Operating Software (GCOS, version 1.4) was used to analyze image data. For each transcript represented on the array by a probe set, the expression algorithm computed the detection call (present, absent, or marginal), the detection p-value, and the signal which is an average intensity value for each probe set. This resulted in a table with 54,675 probe sets. Secondly, for each probe set the 16 detection calls were used to determine whether the probe set was reliably detected or not and should or should not be selected for further analysis (McClintick and Edenberg, 2006). To this end for every group of eight arrays (the early and the non-early) the number of present calls was counted (a number ranging from 0 to 8). If six or more calls were present, the probe set was denoted present. If the probe set was in at least one of the two groups denoted present, it was selected for further analysis. Finally, the over- or underexpression of the remaining probe sets in one of the two groups was analyzed using the class comparison method in BRB ArrayTools software package applying a univariate test composed of a paired T-test with random variance model. This was developed by the Biometric Research Branch of the US National Cancer Institute (*http://linus.nci.nih.gov/BRB-ArrayTools.html*) . Hierarchical clustering of samples was also performed using BRB ArrayTools. Samples were clustered by comparing their expression profiles.

The genes showing significant differential expression between both groups (P<0.01) were classified into functional groups using the Panther classification system (*http://www.pantherdb.org*) (Thomas *et al.*, 2003). The gene expression data analysis tool (Thomas *et al.*, 2006) was used to determine which biological process or pathway was significantly overexpressed in one of the two groups. This program uses binomial statistics with bonferroni correction to analyse whether the proportion of genes from a certain biological process or pathway present in a gene list (i.e. the list of differentially expressed genes from an array study) is significantly different from the proportion of genes in that process or pathway in the whole human genome (*P*<0.05).

Quantitative real-time PCR

For the qRT-PCR, TaqMan low density arrays (TLDAs) (Applied Biosystems, Foster City, USA) were used. Each 2 µl well of the TLDA contains user-defined primers and probes selected from an online catalogue (*http://myscience.appliedbiosystems.com*) for a single gene. One well contains primers and probes for 18S rRNA, a mandatory endogenous control from

the manufacturer. cDNA was prepared from 100 ηg total RNA per sample using the High Capacity cDNA archive kit (Applied Biosystems) according to the manufacturer's instructions. To each cDNA sample (20 µl), 80 µl nuclease-free water and 100 µl 2x TaqMan Universal PCR Master Mix (Applied Biosystems) was added. This mixture was then equally divided over two sample-loading ports of the TLDA, each connected to one set of all the genes of interest. The arrays were centrifuged twice (1', 331g) to equally distribute the sample over the wells. Subsequently, the card was sealed to prevent an exchange between wells. qRT-PCR amplification was performed using an Applied Biosystems Prism 7900HT sequence detection system with the following thermal cycler conditions: 2 minutes at 50°C and 10 minutes at 94.5°C, followed by 40 cycles of 30 seconds at 97°C and 1 minute at 59.7°C.

qRT-PCR analysis

The RQ manager 1.2 software was used to generate Ct values corrected for variances in fluorescent signal strength by using a passive reference dye. The geNorm program (Vandesompele *et al.*, 2002) was used to determine the most stably expressed housekeeping genes. Briefly, the average pairwise variation of a housekeeping gene with all other housekeeping genes was calculated. Stepwise exclusion of the gene with the highest variation resulted in a combination of two housekeeping genes that have the most stable expression. The geometrical mean of the Ct values of these two genes was used as a normalization factor which was substracted from the Ct values of the genes of interest to obtain normalized Ct values (ΔCt). Subsequently, the mean ΔCt of the NEC-CC samples was substracted from the mean ΔCt of the EC-CC samples generating a ΔCt. This ΔΔCt was recalculated into a relative expression quantity ($2^{-\Delta\Delta Ct}$) of the gene of interest in EC-CC as compared with NEC-CC samples (Livak and Schmittgen, 2001).

Results

Microarray analysis

For the gene expression analysis, 8 EC-CC and 8 NEC-CC samples (from 6 patients) have been analysed using 16 microarrays. From the 54,675 probe sets on the array, 18,480 had a present call. Most of these probe sets showed similar expression between the EC-CC and NEC-CC group, except for 737 probe sets that were differentially expressed (P<0.01). For 59 of these probe sets, the corresponding gene is not yet known. Of the 678 remaining probe sets, which correspond to 611 different genes, 162 (24%) were upregulated and 516 (76%) were downregulated in EC-CC compared to NEC-CC. Clustering analysis of the arrays based on the 500

most significantly differentially expressed genes perfectly clustered the EC-CC samples and the NEC-CC samples (Figure 1). The two EC-CC samples derived from the same patient (sample 1-EC a and b and sample 6-EC a and b) and the NEC-CC samples from the same patient (sample 1-NEC a and b and sample 6-NEC a and b) were highly correlated.

Of the 611 genes that are differentially expressed between EC and NEC, 426 could be categorized into one of the biological functions listed in Table I. Processes significantly overrepresented (*P*<0.05, as compared to the whole human genome) according to the Panther gene expression tool are protein modification, nucleic acid, lipid, fatty acid and steroid metabolism, apoptosis, general vesicle transport, cell cycle, structure and motility, chromatin packaging and remodeling, transport and signaling. The relevant pathways in which these genes are involved are Ras, chemokine and cytokine signaling, EGF, FGF, and PDGF receptor signaling and angiogenesis.

In Table II, to economize space, only the genes (n=95) with P<0.001 are categorized per function. Some genes can be categorized into more than one function or process but in Table II they are listed in their most prominent role. Information on the other differentially expressed genes (with P<0.01) can be provided by the authors on request.

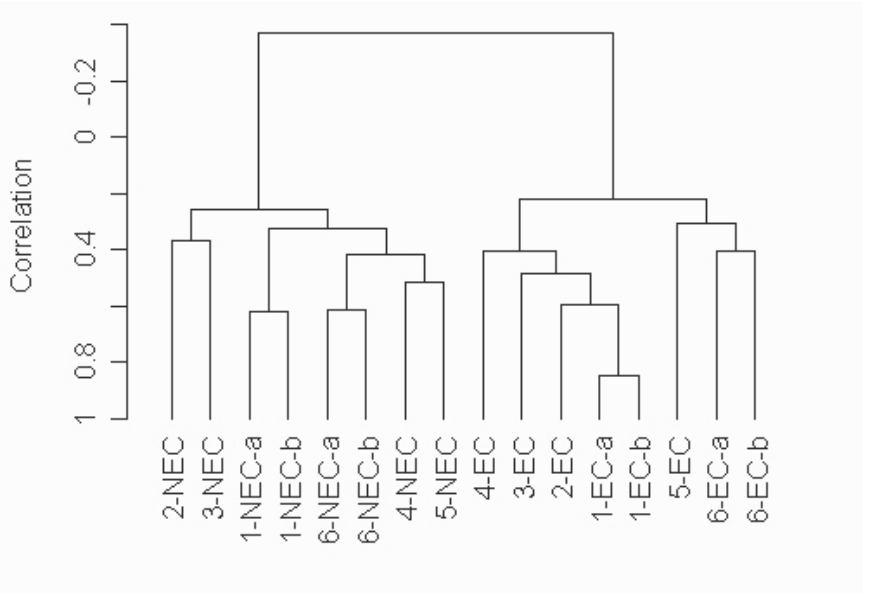

Figure 1: Clustering dendrogram of the 12 samples based on the 500 most significantly differentially expressed genes.

Table I: Number of significantly differentially expressed genes ($P < 0.01$) up or downregulated in EC-CC as compared to NEC-CC, categorized per functional category (% per category)

	Upregulated	(%)	Down regulated	(%)
Antioxidation and free radical removal	0	(0)	3	(100)
Apoptosis	4	(31)	9	(69)
Extracellular matrix, cell communication and cell adhesion	4	(16)	21	(84)
Cell cycle	5	(38)	8	(62)
DNA metabolism, repair and replication	3	(30)	7	(70)
Cell motility and structure	4	(25)	12	(75)
Chromatin packaging and remodeling	2	(20)	8	(80)
Defense	1	(14)	6	(86)
Growth factor	4	(80)	1	(20)
Amino acid metabolism and transport	0	(0)	11	(100)
Carbohydrate metabolism	5	(26)	14	(74)
Lipid, fatty acid and steroid transport and metabolism	4	(22)	14	(78)
Phospholipid metabolism	1	(14)	6	(86)
Oxidative phosphorylation	0	(0)	3	(100)
Protein biosynthese	5	(26)	14	(74)
Protein modification	11	(27)	30	(73)
Proteolysis	5	(17)	25	(83)
Calcium mediated signaling	1	(20)	4	(80)
Cytokine and chemokine mediated signaling	4	(50)	4	(50)
G-protein mediated signaling	3	(23)	10	(77)
Other signaling	7	(21)	26	(79)
Stress response	0	(0)	4	(100)
Transcription factor	10	(20)	40	(80)
mRNA transcription and posttranslational modification	3	(13)	20	(87)
Nucleoside, nucleotide and nucleic acid metabolism	3	(23)	10	(77)
Purine metabolism	2	(22)	7	(78)
RNA processing	0	(0)	3	(100)
Cation transport	4	(33)	8	(67)
Mitochondrial transport	1	(17)	5	(83)
General vesicle transport	3	(25)	9	(75)
Transporter	3	(15)	17	(85)
Other or unknown function	60	(28)	157	(72)

Table II: Genes differentially expressed (P<0.001) in EC-CC vs NEC-CC

Gene ID	Gene description	Probe set	Fold[a]
Antioxidation and free radical removal			
GPX3	glutathione peroxidase 3 (plasma)	201348_at	0.5
PRDX2	peroxiredoxin 2	39729_at	0.7
Apoptosis			
CLU	clusterin (complement lysis inhibitor, SP-40,40, sulfated glycoprotein 2, testosterone-repressed prostate message 2, apolipoprotein J)	208791_at 208792_s_at	0.6 0.6
Extracellular matrix, cell communication and cell adhesion			
CSPG2	chondroitin sulfate proteoglycan 2 (versican)	211571_s_at	0.6
CTNND1	catenin (cadherin-associated protein), delta 1	211240_x_at	0.7
GPC4	glypican 4	204983_s_at	0.6
ITGB1	integrin, beta 1 (fibronectin receptor, beta polypeptide, antigen CD29 includes MDF2, MSK12)	1553678_a_at	0.7
Cell cycle			
76P	gamma tubulin ring complex protein (76p gene)	213266_at	0.8
APRIN	androgen-induced proliferation inhibitor	229704_at	1.3
CCND2	cyclin D2	200953_s_at	0.6
PRC1	protein regulator of cytokinesis 1	218009_s_at	0.6
DNA metabolism, repair and replication			
DKFZP564I0422	THAP domain containing, apoptosis associated protein 2	212202_s_at	0.7
Cell motility and structure			
CFL1	cofilin 1 (non-muscle)	1555730_a_at	0.6
MSN	moesin	200600_at	0.7
PFN1	profilin 1	200634_at	0.6
WASL	Wiskott-Aldrich syndrome-like	205809_s_at	1.6
Chromatin packaging and remodeling			
ARID1A	AT rich interactive domain 1A (SWI- like)	210649_s_at	0.7
Defense			
IFITM1	interferon induced transmembrane protein 1 (9-27)	214022_s_at	0.7
ILF2	interleukin enhancer binding factor 2, 45kDa	200052_s_at	0.7

Amino acid metabolism and transport			
AKAP13	A kinase (PRKA) anchor protein 13	237018_at	0.8
GATM	glycine amidinotransferase (L-arginine: glycine amidinotransferase)	216733_s_at	0.6
Carbohydrate metabolism			
C17orf25	chromosome 17 open reading frame 25	209092_s_at	0.8
PGD	phosphogluconate dehydrogenase	201118_at	0.7
UGP2	UDP-glucose pyrophosphorylase 2	231698_at	1.6
Lipid, fatty acid and steroid transport and metabolism			
ACAD8	acyl-Coenzyme A dehydrogenase family, member 8	221669_s_at	0.7
DHCR7	7-dehydrocholesterol reductase	201791_s_at	0.8
ELOVL5	ELOVL family member 5, elongation of long chain fatty acids (FEN1/Elo2, SUR4/Elo3-like, yeast)	1567219_at	1.5
PLD3	phospholipase D family, member 3	201050_at	0.7
Protein biosynthese			
METAP2	methionyl aminopeptidase 2	209861_s_at	0.7
RPL14	ribosomal protein L14	219138_at	1.5
RPS3	ribosomal protein S3	208692_at	0.8
Protein modification			
DNAJB6	DnaJ (Hsp40) homolog, subfamily B, member 6	208810_at	0.7
OGT	O-linked N-acetylglucosamine (GlcNAc) transferase (UDP-N-acetylglucosamine: polypeptide-N-acetylglucosaminyl transferase)	229787_s_at	1.4
RYK	RYK receptor-like tyrosine kinase	216976_s_at	0.7
SUMO2	SMT3 suppressor of mif two 3 homolog 2 (yeast)	208739_x_at	0.7
Proteolysis			
CST3	cystatin C (amyloid angiopathy and cerebral hemorrhage)	201360_at	0.6
HTRA1	HtrA serine peptidase 1	201185_at	0.7
USP11	ubiquitin specific peptidase 11	208723_at	0.7
XPNPEP1	X-prolyl aminopeptidase (aminopeptidase P) 1, soluble	208453_s_at	0.7
Cytokine and chemokine mediated signaling			
CXCR4	chemokine (C-X-C motif) receptor 4	209201_x_at	0.5
		211919_s_at	0.5

G-protein mediated signaling			
APLP2	amyloid beta (A4) precursor-like protein 2	208703_s_at	0.6
		208248_x_at	0.7
HRB	HIV-1 Rev binding protein	213926_s_at	1.7
Other signaling			
CBL	Cas-Br-M (murine) ecotropic retroviral transforming sequence	229010_at	1.6
LMBR1	limb region 1 homolog (mouse)	224410_s_at	0.7
MAP3K7	mitogen-activated protein kinase kinase kinase 7	211536_x_at	0.8
RAB5B	RAB5B, member RAS oncogene family	201276_at	0.7
Stress response			
HSPB1	heat shock 27kDa protein 1	201841_s_at	0.6
Transcription factor			
NFIB	nuclear factor I/B	209289_at	1.4
PQBP1	polyglutamine binding protein 1	214527_s_at	0.7
TRIM28	tripartite motif-containing 28	200990_at	0.6
TSC22D3	TSC22 domain family, member 3	208763_s_at	0.8
WWTR1	WW domain containing transcription regulator 1	202132_at	1.5
mRNA transcription and posttranslational modification			
DHX9	DEAH (Asp-Glu-Ala-His) box polypeptide 9	212107_s_at	1.5
SNRP70	small nuclear ribonucleoprotein 70kDa polypeptide (RNP antigen)	201221_s_at	0.7
SNRPA	small nuclear ribonucleoprotein polypeptide A	201770_at	0.8
Nucleoside, nucleotide and nucleic acid metabolism			
AK1	adenylate kinase 1	202587_s_at	0.7
BAT1	HLA-B associated transcript 1	200041_s_at	0.7
CSNK2A1	casein kinase 2, alpha 1 polypeptide	229212_at	1.3
DDX3X	DEAD (Asp-Glu-Ala-Asp) box polypeptide 3, X-linked	212515_s_at	0.7
RANBP9	RAN binding protein 9	242143_at	0.7
Purine metabolism			
DKC1	dyskeratosis congenita 1, dyskerin	201478_s_at	0.7
		201479_at	0.7
GUK1	guanylate kinase 1	213621_s_at	1.7
LARP1	La ribonucleoprotein domain family, member 1	212193_s_at	0.6
PRPS2	phosphoribosyl pyrophosphate synthetase 2	230352_at	1.5

Cation transport			
SLC39A9	solute carrier family 39 (zinc transporter), member 9	217859_s_at	1.3
General vesicle transport			
ARF6	ADP-ribosylation factor 6	214182_at	1.6
DCTN1	dynactin 1 (p150, glued homolog, Drosophila)	201082_s_at	0.7
RAB6IP2	RAB6 interacting protein 2	1563947_a_at	1.5
TRAPPC1	trafficking protein particle complex 1	225294_s_at	0.8
Other or unknown function			
AG1	AG1 protein	201104_x_at	0.7
C18orf10	chromosome 18 open reading frame 10	212055_at	0.7
C20orf45	chromosome 20 open reading frame 45	229835_s_at	1.4
C21orf34	chromosome 21 open reading frame 34	239999_at	0.5
C3orf10	chromosome 3 open reading frame 10	224023_s_at	1.6
CKLFSF3	chemokine-like factor superfamily 3	1555705_a_at	0.7
CKS1B	CDC28 protein kinase regulatory subunit 1B	201897_s_at	0.8
CRTAP	cartilage associated protein	1554464_a_at	0.7
DJ328E19.C1.1	hypothetical protein DJ328E19.C1.1	212854_x_at	0.8
EHBP1	EH domain binding protein 1	212650_at	1.4
FOLR1	folate receptor 1 (adult);FOLR1	211074_at	2.2
ILF3	interleukin enhancer binding factor 3, 90kDa	208931_s_at	0.7
KIAA0256	KIAA0256 gene product	212451_at	1.4
KIAA0652	KIAA0652	203364_s_at	0.7
LY6E	lymphocyte antigen 6 complex, locus E	202145_at	0.7
MEIS4	Meis1, myeloid ecotropic viral integration site 1 homolog 4 (mouse)	214077_x_at	1.3
MGEA5	meningioma expressed antigen 5 (hyaluronidase)	223494_at	0.6
NAG8	nasopharyngeal carcinoma associated gene protein-8	210109_at	0.7
PMF1	polyamine-modulated factor 1	202337_at	0.7
RIG	regulated in glioma	221127_s_at	1.5
RTN3	reticulon 3	219549_s_at	0.8
SEL1L	sel-1 suppressor of lin-12-like (C. elegans)	230265_at	1.5
SPTAN1	spectrin, alpha, non-erythrocytic 1 (alpha-fodrin)	208611_s_at	0.7
THOC3	THO complex 3	224623_at	0.7
UST	uronyl-2-sulfotransferase	205139_s_at	0.7
WBP11	WW domain binding protein 11	217821_s_at	0.8

[a] EC-CC:NEC-CC expression ratio

qRT-PCR
From 6 out of 8 NEC-CC samples and from all EC-CC samples sufficient RNA remained for a validation of the microarray results by qRT-PCR. Furthermore, an additional 12 EC-CC and 12 NEC-CC samples were analysed to validate the results in independent samples. qRT-PCR was performed on genes selected either because of their highly significant differential expression in both study groups or because of their involvement in a biological relevant pathway or cellular process (n=25). Furthermore, the most stably expressed housekeeping genes from the microarray (n=4, EIF4G2, PARK7, SRP14 and RHOA) and 18S (a mandatory control by the manufacturer) were included in the qRT-PCR analysis. SRP14 and RHOA were the two most stably expressed housekeeping genes, i.e. the genes with the lowest variation in expression levels. The expression values of the other 25 genes were normalized against the geometrical mean of these two genes.

In the original samples, analysed by microarray, for 22 out of the 25 selected genes (88%) the differential expression between EC-CC and NEC-CC could be confirmed by qRT-PCR (Figure 2). When fold changes between 0.9 and 1.1 were excluded 15 of the 25 (60%) genes could be confirmed. Of these, the differential expression of 10 genes (67%) could be confirmed in the independent samples of which 8 remained after exclusion of the fold changes between 0.9 and 1.1 (Figure 3). These genes were cyclin D2 (CCND2), catenin delta-1 (CTNND1), CXC chemokine receptor 4 (CXCR4), 7-dehydrocholesterol reductase (DHCR7), dishevelled dsh homolog 3 (DVL3), glutathione peroxidase 3 (GPX3), heatshock 27 kDa protein 1 (HSPB1) and tripartite motif-containing 28 (TRIM28).

Discussion

To improve the clinical outcome of eSET, the embryo selection needs to be optimized. Besides the established selection criteria based on embryo morphology and blastomere number, new selection parameters should be developed. Information about the oocyte and its development might be a valuable contribution to the existing selection criteria. As cumulus cells surround the oocyte inside the follicle, a microarray analysis was performed on these cells. Both cumulus cells from oocytes developing into an early cleavage embryo (EC-CC) as well as from oocytes developing into a non-early cleavage embryo (NEC-CC) were compared. Our analysis revealed that 18,480 genes were expressed in cumulus cells, 611 of which showed significant differential expression between EC-CC and NEC-CC. A cluster analysis could perfectly separate the EC-CC and NEC-CC samples, indicating that differences in embryonic implantation potential can already be detected as early as folliculogenesis.

These differences were not manifested in blastomere number and morphology of the embryo as these were similar in both groups.
The differences in gene expression could not be due to differences in age, ovarian stimulation or other patient characteristics as from each patient one EC-CC and one NEC-CC (n=4 patients) sample or two EC-CC and two NEC-CC samples (n=2 patients) were used. By pairing the samples from each patient, the differential gene expression due to different patient characteristics could be ruled out.

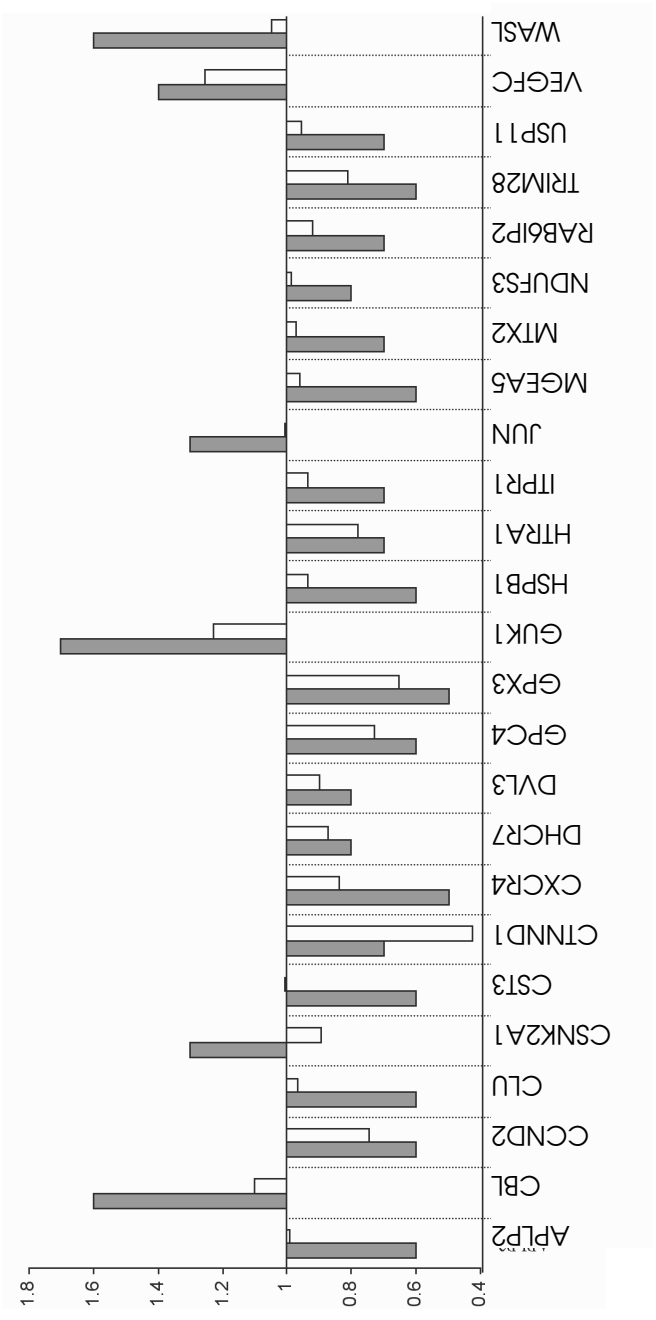

Figure 2: Fold changes (EC-CC vs NEC-CC) of 25 genes measured with microarray analysis (gray bars) and qRT-PCR (white bars) in the same samples.

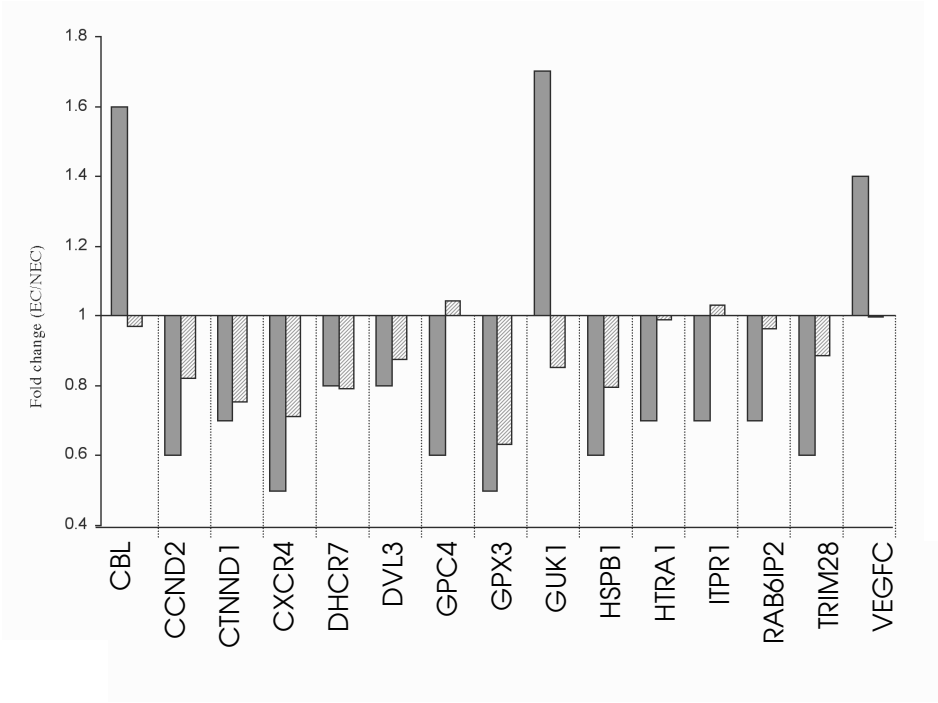

Figure 3: Fold changes (EC-CC vs NEC-CC) of 15 genes measured with microarray analysis (gray bars) and qRT-PCR (striped bars) in different samples.

Furthermore, while other studies analyzing gene expression in human cumulus cells pooled the cumulus cells from several oocytes (Zhang *et al.*, 2005; Assou *et al.*, 2006), in this study each sample consisted of the cumulus cells from one oocyte. This prevented the loss of information.
Of the 611 differentialy expressed genes 24% was overexpressed in EC-CC, while 76% was overexpressed in NEC-CC. The most abundant functions or pathways these genes were involved in were EGF, FGF and PDGF signaling as well as chemokine and cytokine signaling, lipid, fatty acid and steroid metabolism, cell cycle, apoptosis and angiogenesis. Twenty-five genes were selected for validation by quantitative real-time PCR. The gene expression profile found by microarray analysis could be validated for 15 of the 25 (60%) selected genes. In literature, a 84-88% concordance between microarray and quantitative real-time PCR has been described (Rajeevan *et al.*, 2001a; Dallas *et al.*, 2005). Microarray results can be influenced by labeling and hybridization efficiency, while quantitative PCR is dependent on the efficiency of the enzymes and primers.

Especially small fold changes between study groups are sensitive to these variations, which explains the lower concordance in our study (Rajeevan *et al.*, 2001b). The differential expression of 8 of the 15 genes (53%) could be verified in extra independent samples. This indicates that gene expression validation in independent samples is very important to control for genes not consistently over- or underexpressed in the tested conditions and that with microarray analysis alone some genes can show differential expression between the examined conditions by chance. The validated genes are CCND2, CTNND1, CXCR4, DHCR7, DVL3, GPX3, HSPB1, TRIM28.

CCND2 is an important cell cycle regulator. Cell proliferation of cumulus cells is important as a null mutation in Ccnd2 in mice impairs cumulus cell proliferation and leads to small follicles unable to ovulate (Sicinski *et al.*, 1996). In our study the expression of CCND2 is higher in NEC-CC as compared to EC-CC. As the expression of CCND2 is induced by FSH but is subsequently inhibited by LH (Robker and Richards, 1998; Muniz *et al.*, 2006), an increased expression of CCND2 might indicate a diminished sensitivity to LH or a reduced transmission of the LH signaling by the granulosa cells. The increase in CCND2 expression can also be a reaction to compensate for the increase in apoptosis in NEC-CC. In our study, of the 10 differentially expressed genes ($P<0.01$) classified under apoptosis, 8 point to an increase in apoptosis in the NEC-CC samples (6 pro-apoptotic genes were overexpressed and 2 anti-apoptotic genes were underexpressed as compared to EC-CC). An increase in cumulus cell apoptosis has indeed been associated with immaturity of oocytes and an impaired fertilization (Host *et al.*, 2002) and pregnancy rate (Lee *et al.*, 2001). It is not exactly known how these apoptotic signals exert their negative effect on oocyte and embryo development. The apoptotic signals can easily be transferred from the cumulus cells to the oocyte through the gap junctions. After the LH peak, these junctions are however closed. Host *et al.* (2002) suggested, therefore, that the timing of gap junction closure and initiation of apoptotic processes in cumulus cells might be important for oocyte and embryo development.

The chemokine receptor CXCR4 was upregulated in NEC-CC samples. The expression of CXCR4 in cumulus cells was confirmed by Hernandez *et al.* (2006) who localized the protein at the cell surface. CXCR4 in endothelial and cancer cells is expressed via hypoxia inducible factor- (HIF) 1α mediated transcription, which in turn is activated under hypoxic conditions via the phosphatidylinositol 3-kinase (PI 3-kinase) pathway (Schioppa *et al.*, 2003; Staller *et al.*, 2003; Phillips *et al.*, 2005). EGF can also induce PI 3-kinase signaling and upregulate CXCR4 transcription via HIF-1α (Phillips *et al.*, 2005). HIF-1α is a regulator of oxygen homeostasis by functioning as a transcription factor for genes involved in angiogenesis, erythropoiesis, glycolysis and cell proliferation and survival (Semenza, 2002).How CXCR4

can relieve hypoxic stress in cumulus cells needs further investigation.
Another gene which might indicate a hypoxic environment in NEC-CC samples is GPX3. Hypoxia is a strong transcriptional regulator of this gene through its HIF-1 binding sites. It is the only glutathione peroxidase in which these binding sites have been detected (Bierl *et al.*, 2004). Hypoxia leads to the formation of reactive oxygen species (ROS) which can cause lipid peroxidation, enzyme inactivation and cell damage, resulting in apoptosis (Buttke and Sandstrom, 1994) not only in cumulus cells, but also in the oocyte (Tatemoto *et al.*, 2000). Both hypoxia (Van Blerkom *et al.*, 1997) and a concentration of ROS above a certain level in follicular fluid have also been negatively associated with embryonic development, pregnancy outcome (Pasqualotto *et al.*, 2004; Das *et al.*, 2006) and a significantly higher incidence of aneuploidy and spindle defects in oocytes (Chui *et al.*, 1997; Van Blerkom *et al.*, 1997). Nowadays some clinics screen the embryos for aneuploidy by fluorescence in situ hybridization (FISH) on one or two blastomeres biopsied from the embryo (Munne *et al.*, 2003). Although our data need further examination, it would be very promising if intrafollicular hypoxia and thus the enhanced chance for aneuploidy in the corresponding embryo can be analysed by using the cumulus cells instead of removing one or two blastomeres from the embryo.

Although the ultimate goal is to find a new parameter predicting a pregnancy, in this study, EC, which has previously been shown to be a good marker for pregnancy, was used as an endpoint. To correlate a differential gene expression directly to pregnancy, only cycles with SET should be included, as with double embryo transfer (DET) it is not known which embryo implanted. This would however make it impossible to perfectly match samples with a positive and samples with a negative pregnancy outcome for several patient characteristics which probably influence gene expression in cumulus cells. Furthermore, as pregnancy or implantation not only depends on embryonic factors, but also on endometrial receptivity for example, a considerable number of extra microarrays would have been needed in order to find significant intrafollicular differences in gene expression. As EC is a good parameter for pregnancy, independent of blastomere number and morphology (Salumets *et al.*, 2003; Van Montfoort *et al.*, 2004), this was chosen as a marker. Besides, there are indications that whether or not an embryo cleaves early is determined during oogenesis as the human embryonic genome is only activated between the four and eight cell stage (Braude *et al.*, 1988) (Eichenlaub-Ritter and Peschke, 2002). This means that the mature oocyte at ovulation must contain the proteins and mRNA necessary for fertilization and the early stages of embryonic development, including the first cleavage division. Several intrafollicular processes might influence the accumulation of these transcripts. Most investigators of EC concluded indeed after eliminating several explanations for EC like differences in oocyte maturity, that EC might be the result of an

as yet unknown intrinsic oocyte factor (Shoukir *et al.*, 1997; Sakkas *et al.*, 1998; Lundin *et al.*, 2001; Fenwick *et al.*, 2002).
In conclusion, this study provides evidence that embryo viability is reflected in differential gene expression in the cumulus cells. The molecular discrimination of cumulus cells from different oocytes might lead to an improved embryo selection with improved eSET results or might serve as a tool for oocyte selection necessary in countries where not all oocytes are allowed to be fertilized.

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CHAPTER 8

General discussion

The number of IVF treatments of subfertility throughout Europe is still increasing, as can be learned from the annual ESHRE reports on IVF in Europe (Nyboe Andersen *et al.*, 2004; 2005; 2006). In Finland and Denmark, nowadays 2.9 and 4.2% of the newborn children are conceived by IVF (Nyboe Andersen *et al.*, 2006). For The Netherlands this figure can only be estimated since no national registry exists for children conceived by IVF. In 2005, 4725 pregnancies were obtained by IVF and ICSI procedures, leading to 3653 ongoing pregnancies (www.nvog.nl). If we estimate these to have led to 3500 live births, of which 81.3% singletons, 18.5% twins and 0.2% triplets (www.nvog.nl), this would result in 4162 children of which 32% was part of a twin or triplet pregnancy. In 2005, in total 187,910 children were born (statline.cbs.nl), so in The Netherlands 2.2% of the children were the result of IVF. This IVF procedure is however not without risks and complications, the most important ones being those secondary to multiple pregnancies following multiple embryo transfer. In order to reduce this multiple, and especially twin pregnancy rate (PR), the aim of the thesis was to compare several transfer strategies with eSET from a clinical and a cost-effectiveness point of view and to improve the selection of the single best embryo for eSET.

eSET – clinical outcome

After a first conservative application of eSET in patients with only one embryo available, medical reasons demanding SET, or patients wishing the transfer of one embryo only (Vilska *et al.*, 1999; Tiitinen *et al.*, 2001), eSET was applied more generally, but always based on female age, embryo quality and cycle rank (Gerris *et al.*, 2002; De Sutter *et al.*, 2003). The limits of these criteria for eSET varied among several studies (see Tables I and II).

In our centre, instead of gradually liberating female age and embryo quality limits for eSET, we investigated whether eSET could be applied regardless of these criteria by performing an RCT on eSET vs DET in the first cycle of an unselected group of patients (chapter 3). The transfer of one embryo did indeed result in a significant reduction of the twin PR, but at the expense of a 50% reduction in ongoing PR. Compared to other RCTs in selected patients, the eSET PR in our study was lower, but the difference in PR between eSET and DET was found in all RCTs (Table I). This indicates that the transfer of two embryos will always lead to a higher PR as compared to the transfer of only one embryo.

In several studies the application of an eSET/DET policy, i.e. eSET in a selected good prognosis population and DET in the remaining patients, was analysed (Table II). Despite the liberalization of the limits for eSET, the PR after eSET and DET remained similar. Most clinics apply such an eSET/DET policy only in cycle one and two (Tiitinen *et al.*, 2003; Gerris *et al.*, 2004;

Martikainen *et al.*, 2004). From chapter 2 it can be concluded that such a policy is also suitable for the third cycle. Although as expected, the PR in the third cycle was lower as compared to the PR in the first and second cycle, the PR after eSET and DET was similar per cycle. The cumulative overall twin PR in three fresh cycles eSET and DET in the study period July 2000 – December 2001 was still 22.4%, probably due to the fact that the proportion of cycles with eSET was relatively small; 21% of the cycles with more than one embryo available. After a less stringent selection for eSET in the RCT study-period 2002-2004, in 43% of the fresh cycles one embryo could be transferred (chapter 5), leading to an ongoing PR of 52.9% and a twin PR of 12.9% after a maximum of three fresh cycles eSET/DET.

Although with an eSET/DET policy the PR in the two groups is similar, when two embryos would be transferred in the eSET group, the PR would be higher. To compensate for this difference other strategies were investigated. In chapter 5 besides eSET in the first cycle of all patients, an eSET/DET policy was applied in the second and third cycle. This led to a similar cumulative PR as compared to three cycles eSET/DET, but also to a similar cumulative twin PR, annulling the motive for applying eSET. According to Lukassen *et al.* (2005) a second eSET cycle compensates for the decrease in PR in the first cycle. In an RCT on eSET vs DET in their selected patient population (Table I), the live birth rate was 26% after eSET and 36% after DET.

When the non-pregnant patients in the eSET group received a second eSET cycle, the cumulative live birth rate increased to 41%. With this strategy, the twin PR remained reduced, but at the expense of an extra cycle for the patients. Thurin *et al.* published another solution for the reduction in PR, a subsequent cycle with a single frozen-thawed embryo, if available from the initial eSET cycle. After the fresh eSET or DET cycle, the ongoing PRs were 28.5% vs 44.1%, respectively (P <0.001). When subsequently a single frozen-thawed embryo was transferred in the eSET group, the ongoing PR increased to 37.8% (compared to 44.1% after one fresh DET cycle, P = 0.07) (Thurin *et al.*, 2004). The contribution of cryopreserved embryos to the PR after eSET was also confirmed by Tiitinen *et al.* (2001). However, in both studies it was not taken into account that after DET also embryos were cryopreserved.

Table I: Selection criteria and pregnancy outcome in RCTs on one cycle eSET versus one cycle DET (fresh embryos)

	Selection criteria for RCT				eSET		DET	
	age	No of good qualityembryos	Cycle	*n*	Pregnancy rate (%)	Twin rate (%)	Pregnancy rate (%)	Twin rate (%)
Gerris *et al.*, 1999	< 34	≥ 2	1	53	10/26 (38.5)[a]	1/10 (10.0)	20/27 (74.1)[a]	6/20 (30.0)
Martikainen *et al.*, 2001[1]	-	≥ 4	1 or 2	101	22/74 (29.7)	1/22 (4.5)	28/70 (40.0)	11/28 (39.2)
	< 36	≥ 4	1	43				
Gardner *et al.* 2004[3]	< 45			48	14/23 (60.9)	0/14 (0)[b]	19/25 (76.0)	9/19 (47.4)[b]
Thurin *et al.*, 2004[2]	< 35	≥ 3	1 or 2	215	94/330 (28.5)[a]	1/94 (1.1)[b]	146/331 (44.1)[a]	47/146 (32.2)[b]
	< 36	≥ 2	1 or 2	446				
Lukassen *et al.*, 2005	< 35	≥ 2	1	107	14/54 (25.9)	0/14 (0)[b]	19/53 (35.8)	7/19 (36.8)[b]
Van Montfoort *et al.* 2006	< 41	-	1	308	33/154 (21.4)[a]	0/33 (0)[b]	62/154 (40.3)[a]	13/62 (21.0)[b]

[1] multicenter trial with some centres having aberrant criteria

[2] After the first 215 patients the criteria were changed due to a change in the usual clinical practice in Sweden

[3] Study on single blastocyst transfer vs double blastocyst transfer with no embryo or cycle limits

[a] Pregnancy rates within one row are significantly different ($P < 0.05$)

[b] Twin pregnancy rates within one row are significantly different ($P < 0.05$)

Table II: Selection criteria for eSET and pregnancy outcome in observational studies on eSET and DET (fresh embryos)

	Selection criteria for eSET	n	eSET (%)	eSET		DET		Overall	
				Pregnancy rate (%)	Twin rate (%)	Pregnancy rate (%)	Twin rate (%)	Pregnancy rate (%)	Twin rate (%)
Vilska *et al.* 1999	Wish or medical reasons	816	9.1	22/74 (29.7)	0/22 (0)	218/742 (29.4)	52/218 (23.9)	240/816 (29.4)	52/240 (21.6)
Tiitinen *et al.* 2001	Wish or medical reasons	644	19.7	49/127 (38.6)	1/49 (2.0)	203/517 (40.0)	42/203 (20.7)	252/644 (39.1)	43/252 (17.1)
Tiitinen *et al.* 2003[a]	≥ 1GQE 1st or 2nd cycle	537	56.1	102/301 (33.9)	NA	93/236 (39.4)	NA	195/537 (36.3)	29/195[b] (14.9)
Gerris *et al.* 2004	< 38 y Wish ànd ≥ 1GQE 1st cycle	367	56.1	83/206 (40.3)	0/83 (0)	65/161 (40.4)	20/65 (30.8)	148/367 (40.3)	20/148 (13.5)
Martikainen *et al.* 2004	< 36 y ≥ 1 GQE 1st or 2nd cycle	1111	27.7	107/308 (34.7)	1/107 (0.9)	255/803 (31.8)	NA	362/1111 (32.6)	NA

Van Montfoort *et al.* 2005[c]	< 38 y ≥ 1 GQE 1st, 2nd or 3th cycle	326	21.3[c]	35/111 (31.5)	0/35 (0)	119/410 (29.0)	38/119 (31.9)	154/521 (29.6)	38/154 (24.7)
Van Montfoort *et al.* 2006	< 38 y ≥ 1 GQE 1st cycle	222	45.0	33/100 (33.0)	1/33 (3.0)	37/122 (30.3)	8/37 (21.6)	70/222 (31.5)	9/70 (12.9)
Veleva *et al.* 2006[d]	36 – 39 y ≥ 1 GQE	920	36.4	111/335 (33.1)	0/111 (0)	175/585 (29.9)	31/175 (17.7)	286/920 (31.1)	31/286 (10.8)

[a] Data from 2000 and 2001
[b] number of twin pregnancies estimated from bar chart
[c] Instead of one cycle per patient, a maximum of three subsequent cycles was analysed. %eSET is per total number of cycles
[d] This study was performed in women aged 36-39 years only
GQE= good quality embryo
y= years (female age)

Perspectives on eSET

As described above, in some clinics all efforts were focussed on maximizing the eSET rate and on how eSET matches optimally in a transfer policy. However, considering the opinions of several actors involved in policy making like the medical practitioners, patients, politicions, health economists and embryologists, it is debatable whether eSET in the majority of patients is the appropriate solution. Opinions from these different actors will be discussed.

Medical perspective

For the medical practitioners a conflict exists. On one hand, the rate of patients with a live birth after IVF should be as high a possible, which can be achieved by DET. On the other hand IVF clinicians feel also responsible for the well-being of the offspring. The number of multiple pregnancies and the complications thereof should be as low as possible, favouring eSET. As several clinics already apply eSET in a selected group of patients (ranging from 6.9% in Ireland to 38.7% in Finland in 2002 (Nyboe Andersen *et al.*, 2006)), medical practitioners indirectly indicate that they accept a lower PR at the benefit of preventing twin pregnancies.

According to the meta-analysis of RCTs on eSET vs DET in a selected group of patients performed by Pandian *et al.* (2005), medical practitioners accept a live birth rate reduction of 15% in the patients receiving one embryo by accepting an eSET/DET policy. Assuming that in 50% of the patients eSET is applied, which is common in some clinics (Tiitinen *et al.*, 2003; Debrock *et al.*, 2005), the live birth rate for the total IVF population will be reduced with 7.5% (0.50x0.15) and the number of twins will be halved. What is the maximal reduction in PR acceptable for the practitioners?

Based on the conclusions made after observational studies on eSET in a selected group of patients and DET in the remaining part, IVF clinicians are satisfied when the PR is comparable in both groups (Tiitinen *et al.*, 2003; Gerris *et al.*, 2004; Martikainen *et al.*, 2004). However, when the selection limits for eSET are liberalised (e.g. an increased age limit or less strict criteria for a good quality embryo), the proportion of eSET will increase, probably resulting in a decrease in PR. As the pregnancy prognosis of the DET group will decrease as well, this again might result in a comparable PR after eSET and DET, but at a lower level. At which level will this be no longer acceptable? Worldwide, in the majority of clinics, still two or even more embryos are transferred, indicating that in these clinics the number of live births gets priority above the well-being of the offspring. Performing DET in all patients would result in a live birth rate of 39.6% (chapter 4), while in the normal fertile population only 30% of the conceptions result in a live birth (Macklon *et al.*, 2002). It can be argued whether subfertile patients should be made 'better' than the fertile population. Most of the 'healthy' population achieves a pregnancy from a single embryo. Apparently, as a

rule, in the human, nature provides for singleton pregnancies. Why should subfertile patients then achieve a pregnancy from multiple embryos? In addition, recent studies show that not only twins born after DET, but also singletons born after DET are at an increased risk of an adverse obstetric outcome (birth weight and gestational age) (Pinborg *et al.*, 2005; De Sutter *et al.*, 2006). This is correlated with the onset of the spontaneous reduction of a twin pregnancy to a singleton pregnancy (i.e. a so-called 'vanishing twin') (Pinborg *et al.*, 2005).
By transfer of only one embryo in the subfertile population, a live birth rate of 20.8% per cycle can be achieved (chapter 4) with a maximal dizygotic twin PR reduction (to 0%). As this is not applied in any clinic and in most clinics still two or even more embryos are transferred, practitioners or the possibly more decisive actors described below, apparently have a limit of the reduction in overall PR they accept in order to prevent twin pregnancies and their complications.

Patients' perspective
A study by Blennborn *et al.* (2005) on the decision-making between eSET and DET in IVF patients revealed that previous childbirth and the availability of cryopreserved embryos were the most important variables in deciding pro-eSET. Previous failed IVF treatments and an assumed increased PR after DET were variables positively influencing the patients' choice for DET. The importance of PR for patients was confirmed by Murray *et al.* (2004). According to the answers on a questionnaire in patients starting their first cycle, 30% of the patients would accept eSET when the PR would be slightly reduced, while > 80% of the patients would accept eSET when PR remained unchanged. Furthermore, although a twin pregnancy is not regarded as an undesired outcome (67-90% would not have mind to conceive twins, (Gleicher *et al.*, 1995)), a minority of the patients prefers a twin pregnancy above a singleton pregnancy (20.3% (Ryan *et al.*, 2004); 41% (Child *et al.*, 2004)). The desire for a twin is associated with nulliparity, younger patient age, limited knowledge about outcomes of twin pregnancy, lower income and longer duration of infertility (Ryan *et al.*, 2004). Improving the knowledge of patients about the risk and complications of twin pregnancies by an educational campaign enhanced the preference of patients for a singleton pregnancy and for SET (Ryan *et al.*, 2006). However, this theoretical desire for eSET was not translated into action. When these patients were asked to actually have one embryo transferred during their treatment no increase in preference for eSET was seen (Ryan *et al.*, 2006). In contrast, in a study by Gerris *et al.* (2004), 66% of the patients chose for an eSET/DET policy instead of DET. Although the PR is similar between eSET with a good quality embryo or DET with moderate quality embryos, when a patient with good quality embryos receives DET the overall PR would be higher. Therefore, it should however be taken into account that studies on patients preferences depend largely on how patients are informed.

Also reimbursement of an IVF treatment influences the preference for eSET or DET as the preference for eSET increased from 30% to 50% when patients were theoretically not charged for any subsequent frozen embryo transfer cycles after fresh eSET (Murray *et al.*, 2004).
The importance of PR and reimbursement for the acceptance of eSET by the patient can also indirectly be concluded from chapter 3. After counseling, a majority of the patients (56%) agreed to participate in the RCT study on eSET versus DET, where an extra fourth cycle free of charge was offered to compensate for a possible decrease in PR in the eSET arm of the study.

Political perspective
Health authorities can state their preference for the number of embryos to be transferred through imposing laws on several issues regarding IVF. For instance, in Germany and Italy an oocyte selection has to be made as all produced embryos have to be transferred. The number of embryos may not exceed three and the cryopreservation of supernumerary embryos is prohibited (Benagiano and Gianaroli, 2004). An eSET policy is therefore discouraged. The legislation on insurance coverage of IVF can also (dis)courage eSET. The compensation for the reduction in PR after eSET by an additional cycle will be more easily accepted when these cycles are reimbursed (see patients' perspective). France for example has an unlimited coverage, while almost half of all countries has no coverage (IFFS Surveillance 04, 2004). The most direct manner for health authorities to influence the number of embryos to be transferred is to impose a law on that issue. As the maximum number of embryos to be transferred is subject to wide variation among countries, the political perspective regarding eSET and DET is not uniform (IFFS Surveillance 04, 2004). In some countries the legislation is adjusted to eSET. In Belgium for example, the general aim of the government was to make fertility treatment accessible for more patients with the same financial resources. After a proposal from all IVF centres, besides the medical costs also the laboratory costs are reimbursed on the condition that in the first cycle of all patients < 35 years eSET is applied (Ombelet, 2004). According to the proposal, the costs of the procedure balance the reduction in costs due to the reduction in twin pregnancies (De Sutter and Gerris, 2006).
In Sweden, the legislation prescribes mandatory eSET, with the exception that two embryos may be transferred if the twinning risk is low (Thurin *et al.*, 2004). This was based on the encouraging reports on eSET, the demands by neonatologists, and for economical reasons (Saldeen and Sundstrom, 2005). The decree is however subject to clinical variation, as the attitude of the clinics towards twinning risk differs.
With only two countries imposing a legislation regarding eSET, politics is not convinced already. Remarkable, the legislation in these countries was changed after a thorough discussion between the authorities and

the IVF clinicians and neonatologists. This would suggest that politicians in some countries have not changed legislation due to a lack of information and that by informing them about the complications involved in twin pregnancies, they can be convinced to change legislation.

Health economic perspective

From a health economist's point of view the transfer policy with the lowest costs per live birth is preferred. When regarding an eSET or a DET policy, the difference in cost-effectiveness can be attributed to a difference in live birth rates and costs. The latter is mainly caused by the twin pregnancies in the DET group requiring more examinations and care during the antenatal period, more loss of productivity, an increase in maternal and neonatal hospitalization and considerably higher long term costs (Gerris *et al.*, 2004; Lukassen *et al.*, 2004; Thurin *et al.*, 2004) plus the extra number of cycles in the eSET group necessary for achieving a pregnancy. The most favourable cost-effective strategy will be the one in which the costs of additional cycles in the eSET strategy will balance the costs of a twin pregnancy in the DET strategy.

As described in chapter 4, when eSET is applied in all patients, irrespective of age and embryo quality, the total costs per patient up to 42 weeks after embryo transfer are € 7,334 with a live birth rate of 20.8%. This results in € 35,260 (7334/0.208) per live birth. A DET strategy in these patients costs € 10,924 and with a live birth rate of 39.6% this strategy would cost € 27,586 (10924/0.396) per live birth. The ICER of DET compared with SET was € 19,096, meaning that each additional live birth in the DET group, as compared to the eSET group, would cost € 19,096. It depends on what amount of money is regarded as acceptable for an extra live born child to decide whether DET is preferred above eSET from a cost-effectiveness point of view. When eSET or DET was applied in a selected group of patients and if an additional frozen-thawed eSET cycle were to be performed in case no live birth was achieved after eSET, the costs were € 9,309 and € 12,318 for an eSET and DET strategy (Kjellberg *et al.*, 2006). With live birth rates of 38.8% and 42.9% this resulted in € 23,992 and € 28,713 per live birth after eSET and DET, respectively. The ICER of DET compared with SET was € 73,307, putting DET at a disadvantage (Kjellberg *et al.*, 2006).

eSET in all patients is not desirable from a health economic point of view. In a selected good prognosis population with an additional frozen-thawed cycle eSET is preferrable to DET. The remaining population has not been taken into account. Therefore it is necessary to calculate whether eSET in the selected good prognosis patients and DET in the remaining patients is still more cost-effective than DET in all patients. Another limitation of all cost-effectiveness studies is that the long-term consequences of eSET and DET are not taken into account. It is obvious that these costs are higher after DET than after eSET, as twins suffer more developmental problems and handicaps than singletons (Luke and Keith, 1992).

Embryologists' perspective
The challenge of eSET for an embryologist is to select the most appropriate embryo for transfer. Mostly, this selection is based on morphological criteria. At all stages of development criteria were defined to be fulfilled by the qualitatively best embryos (see Borini *et al.* (2005) for review). The number of blastomeres and morphological aspects are commonly used. The predictive value of some 'new' parameters, like oocyte and polar body I morphology remain controversial (Borini *et al.*, 2005). However early cleavage, the first cleavage of an embryo before 23-26h after injection or before 25-28h after insemination, seems even in single embryo transfers unequivocaly to be a strong predictor, independent of cell number and embro morphology (chapter 6).
In embryo selection it is not only the morphological appearance that characterizes a good quality embryo. Also from within the embryo important information can be obtained, e.g. the metabolic activity of an embryo can be examined by analysing the uptake of nutrients from or the secretion of metabolites into the surrounding medium (Gardner *et al.*, 2001; Van den Bergh *et al.*, 2001). Also the presence of chromosomal aberrations in embryos is a prognostic factor. By removing one or two blastomeres from the embryo, the ploidy state can be determined in these cells by fluorescent in situ hybridization (FISH). Besides that this method is very invasive and detrimental to the embryo, its effectiveness for improving ongoing PR is also being questioned (Twisk *et al.*, 2006)

Therefore a new examination method was put forward: gene expression in cumulus cells (chapter 7). Cumulus cells from oocytes resulting in an early or non-early cleavage embryo with similar morphology grades and cell numbers on the second day of development display a differential gene expression. Although this should be developed further, it indicates that this gene expression may become a valuable marker for embryo quality which is not reflected in the embryo morphology and blastocyst number. Gene expression in cumulus cells might also become a valuable tool for those clinics that have permission to fertilize only a limited number of oocytes. By analysing the cumulus cells, the oocytes with a high embryonic developmental potential can be selected. Hamamah *et al.* (2006) identified several proteins that are differentially expressed in cumulus cells depending on fertilization of the oocyte. Other studies focus for instance on the concentration of HLA-G in culture medium or the secretion of proteins at a different level between embryos with and without chromosomal defects (Pearson, 2006). Although these are all new methods that need further investigation, they are all non-invasive for the embryo and can lead to a better embryo selection and therefore a better outcome with eSET.

Summary and conclusion

Nature has meant the human female to carry singleton pregnancies. The uterus, the cardiovascular system and metabolism are geared towards singletons. To prevent twin pregnancies in IVF, an increasing tendency exists to apply eSET. However, when taking together all factors influencing an eSET policy, it appears that at the moment eSET is not suitable for every patient. Although medical practitioners accept a certain PR reduction for the benefit of creating less twin pregnancies, there is a limit to what is deemed acceptable. This limit is however yet to be defined and apperently differs from country to country (Gleicher *et al.*, 2006).

The patients, on the other hand, want the highest chance of a pregnancy, which would suggest DET. Only when the PR is not reduced, they are prepared to accept an eSET/DET policy. Finally, from a cost-effectiveness point of view, eSET in all patients is not preferable to DET. The cost-effectiveness of an eSET/DET policy will be analysed in a companion thesis from our group. For the future, cost-effectiveness analyses should also take the long-term costs of handicaps resulting from twin pregnancies into account since these might ultimately determine the place of assisted reproduction in our health care system.

In conclusion, based on the data obtained so far, an eSET/DET policy is preferable at this moment. The question on what should be the criteria for eSET remains to be solved. In general, a balance has to be found between the decrease in PR with eSET and the decrease in twin PR. A small decrease in PR as compared to DET should be associated with a large decrease in twin PR. It should be carefully considered whether measures decreasing the twin PR only marginally, balance the reduction in PR as only a certain percentage of the twins has complications that should be prevented. Furthermore, a cost-effectiveness analysis should be applied on several transfer strategies, including an eSET/DET policy and including the long-term costs for twins. The patients' knowledge about the risks of a twin pregnancy should be enhanced to increase the acceptance of eSET, at least in a good prognosis subpopulation. And finally, the effectiveness of new non-invasive embryo selection techniques should be examined in order to enhance the PR after eSET.

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Summary

A twin pregnancy is, due to its complications, regarded a disadvantageous outcome of an IVF treatment. For the Netherlands an estimated 2.2% of all newborn children is conceived by IVF. Of these, 32% is part of a twin. The complications, like hypertension in the mother and preterm delivery and low birth weight of the children, lead to high health care costs, which increase even more when the costs for life-long handicaps and developmental problems are included. The twin pregnancies result from the transfer of two or more embryos per cycle. By transferring only one embryo (single embryo transfer, SET) twin pregnancies can be avoided. The aim of this thesis was to compare several SET transfer strategies from a clinical and cost-effectiveness point of view, and to improve the selection of the single best embryo for SET.

In chapter 2, a transfer policy is described for patients with at least two embryos available. Patients younger than 38 years with at least one embryo of good quality received one embryo (around 20% of the patients). Patients of 38 years or older or patients with moderate quality embryos received two embryos (DET, 80% of the patients). In the first as well as the second and third treatment cycle, a statistically similar pregnancy rate was obtained for both groups (32.7 and 33.2%; 36.1 and 22.9%; and 20.0 and 24.2%, respectively). Because in significantly more SET cycles embryos could be frozen, the cumulative pregnancy rate after the transfer of both fresh and, when appropriate, frozen embryos was significantly higher after SET as compared to DET. However, the overall twin pregnancy rate was still 22.9%.

In a subsequent time-period, the above mentioned transfer strategy was applied in the first cycle with liberalized embryo selection criteria. This resulted in 45% SETs and 55% DETs. The pregnancy rates were similar again (33.0 and 30.3%, respectively) and the overall twin pregnancy rate was 12.9% (chapter 3).

To abolish twin pregnancies, all IVF patients should receive one embryo. In a randomized controlled trial, described in chapter 3, in which patients with at least two embryos available were randomized for their first cycle between SET or DET irrespective of their age and of the embryo quality, it appeared that the pregnancy rate would decrease significantly if SET was applied in all cycles (pregnancy rate was 21.4% after SET and 40.3% after DET). The twin pregnancy rate was reduced from 21.0% after DET to 0% after SET. Alongside this clinical trial, a cost-effectiveness analysis on both strategies (SET or DET in all IVF patients, chapter 4) was performed. The costs of an IVF treatment including the costs of a possible pregnancy and delivery (until 4 weeks after delivery) were on average €7334 for SET

and €10924 for DET. This difference was mainly caused by the augmented costs for pregnancy and delivery in the DET strategy. This more expensive strategy however, also led to more successful pregnancies. Each additional successful pregnancy obtained with a DET strategy as compared to the SET strategy cost €19096 extra.
Finally, in chapter 5 both transfer strategies were combined. All patients younger than 38 years with at least two embryos available received one embryo in their first cycle. If in the second or third treatment cycle one embryo of good quality was available SET and otherwise DET was applied (group A). In the control group (group B), the transfer strategy for the first cycle was similar to that in the second and third cycle; SET when at least one embryo of good quality was available and otherwise DET. The live birth rates after 3 cycles were similar in both groups (62.4 and 62.6%, respectively). However, the twin pregnancy rate was not reduced by transferring one embryo in the first cycle of all IVF patients (10.1% group A and 13.4% group B). Furthermore, group A required significantly more fresh (2.0 vs 1.8) and frozen cycles (0.8 and 0.5) to achieve a similar pregnancy rate than group B.

To increase the pregnancy rate with SET, more information is needed about the selection criteria for an embryo with a high implantation potential. Besides the established cell number and embryo morphology on the day of transfer, the timing of the first cleavage division seemed important (chapter 6). Embryos that cleaved to the two-cell stage between 23-26h post injection (ICSI) or 25-28h post-insemination (IVF), implanted significantly more often as compared to late cleaving embryos. The transfer of a single early cleavage embryo resulted in 37.1% of the patients in a pregnancy while this was the case in only 10.3% of the patients receiving a non-early cleavage embryo. A logistic regression analysis revealed that early cleavage was a predictor of pregnancy and blastocyst formation independent of cell number and embryo morphology.

Morphological aspects are insufficient to characterize a good quality embryo. Also from within the embryo or the oocyte important information can be obtained. In chapter 7, a study is described in which the genome-wide gene expression in cumulus cells was analysed. These cells surround the intra-follicular oocytes. Differences in intrafollicular conditions important for oocyte and embryo development might be reflected in differences in expression of several genes in cumulus cells. Microarrays were used to analyse genome-wide differential gene expression in cumulus cells from oocytes resulting in good and moderate quality embryos. The expression of 611 genes, among others involved in cell cycle, angiogenesis, apoptosis, growth factor, chemokine and cytokine signaling and hypoxia, was related to embryo quality.

Although further research is needed, these results might point towards a stress-inducing environment for oocytes that result in moderate quality embryos.
In chapter 8 the results are discussed and the opinion of several actors involved in transfer policy making, like medical practitioners, patients, politicians, embryologists and health economists is described. In conclusion, a SET strategy is at the moment not suitable for all patients, but only for a selected group of top quality patients with top quality embryos. The exact selection criteria, not only embryo selection criteria but also patient selection criteria need to be further developed.v

Samenvatting

Een tweelingzwangerschap wordt, vanwege de bijbehorende complicaties, gezien als een nadeel van een IVF behandeling. In Nederland is naar schatting 2,2% van de pasgeboren kinderen het resultaat van IVF. Hiervan behoort 32% tot een tweeling. De complicaties, zoals hypertensie bij de moeder en vroeggeboorte en verlaagd geboortegewicht bij de kinderen leiden tot hoge kosten in de gezondheidszorg. Deze kosten worden nog hoger als ook de kosten van levenslange ontwikkelingsproblemen en chronische ziektes ten gevolge van de tweelingzwangerschappen meegenomen worden. De tweelingzwangerschappen ontstaan doordat vaak meer embryo's tegelijk terug geplaatst worden. Door maar één embryo terug te plaatsen (=single embryo transfer, SET) zouden tweelingzwangerschappen voorkomen kunnen worden. Het doel van dit proefschrift was dan ook het analyseren van verschillende SET strategieën vanuit een klinisch en kosten-effectief oogpunt en het verbeteren van de selectie van het embryo met de hoogste implantatie kans.

In hoofdstuk 2 wordt een terugplaatsstrategie beschreven voor patiënten met tenminste 2 embryo's. Patiënten jonger dan 38 jaar met tenminste 1 embryo van goede kwaliteit kregen één embryo terug (ongeveer 20% van de patiënten). Bij patiënten van 38 jaar en ouder en patiënten met minder goede embryo's werden twee embryo's teruggeplaatst (DET, 80% van de patiënten). Zowel in de 1e als in de 2e en 3e behandelcyclus was de zwangerschapskans statistisch vergelijkbaar voor SET en DET (respectievelijk 32,7 en 33,2%, 36,1 en 22,9%, en 20,0 en 24,2%). Omdat na SET significant vaker embryo's konden worden ingevroren, was het cumulatieve zwangerschapspercentage na de terugplaatsing van zowel verse als ingevroren embryo's na SET significant hoger dan na DET. Met deze terugplaatsstrategie was het tweelingzwangerschapspercentage van alle IVF zwangerschappen echter nog steeds hoog, namelijk 23%.
In een volgend cohort werd bovenstaande terugplaatsstrategie herhaald voor de 1e cyclus, waarbij de criteria voor een kwalitatief goed embryo werden versoepeld. Hierdoor kregen 45% van de patiënten één embryo terug en 55% twee. De zwangerschapskansen waren wederom vergelijkbaar voor SET en DET (respectievelijk 33,0 en 30,3%) en het tweelingzwangerschapspercentage was 12,9% voor de hele IVF groep (hoofdstuk 3).
Om het tweelingzwangerschapspercentage zo laag mogelijk te krijgen zou bij àlle IVF patiënten één embryo teruggeplaatst moeten worden. Uit een gerandomiseerde studie, beschreven in hoofdstuk 3, waarin alle patiënten met tenminste 2 embryo's in hun eerste cyclus werden gerandomiseerd tussen SET of DET, ongeacht leeftijd en ongeacht embryo kwaliteit, bleek echter dat de kans op zwangerschap significant zou dalen als bij àlle IVF patiënten één embryo teruggeplaatst zou worden

(zwangerschapskans was 21,4% na SET en 40,3% na DET). Het percentage tweelingzwangerschappen werd teruggebracht van 21,0% na DET naar 0% na SET. Parallel aan deze klinische studie werd een kosteneffectiviteits analyse gedaan van beide strategieën (SET of DET in alle patiënten, hoofdstuk 4). De kosten van een IVF behandeling inclusief de kosten van een eventuele zwangerschap tot 4 weken na de bevalling waren gemiddeld €7334 voor de SET strategie en €10924 voor de DET strategie. Dit verschil werd voornamelijk veroorzaakt door de hogere kosten tijdens zwangerschap en bevalling na DET. Deze duurdere strategie leidde echter ook tot meer succesvolle zwangerschappen. Elk levend geboren kind dat extra geboren werd na DET ten opzichte van eSET kostte €19096.

Als laatste werden in hoofdstuk 5 beide terugplaatsstrategieën gecombineerd. Patiënten jonger dan 38 jaar met minimaal 2 embryo's kregen allemaal SET in de eerste cyclus. Als er in de 2e of 3e cyclus een embryo van goede kwaliteit aanwezig was, kregen ze ook dan SET. Waren in de 2e of 3e cyclus alleen embryo's van mindere kwaliteit beschikbaar, dan werden er twee embryo's teruggeplaatst (groep A). Voor de controle groep (groep B, ook patiënten jonger dan 38 jaar met tenminste 2 embryo's beschikbaar) was de 1e cyclus gelijk aan cyclus 2 en 3; alleen SET als er een embryo van goede kwaliteit aanwezig was, anders DET. De cumulatieve kans op een levend geboren kind na 3 cycli was in beide groepen even hoog (respectievelijk 62,4 en 62,6%). Echter het percentage tweelingzwangerschappen werd niet verlaagd door bij iedereen in de 1e cyclus één embryo terug te plaatsen (10,1% voor groep A en 13,4% voor groep B) en bovendien moest deze groep meer puncties (respectievelijk 2.0 en 1.8) en meer vries-dooicycli (respectievelijk 0.8 en 0.5) ondergaan om tot hetzelfde zwangerschapspercentage te komen.

Om de zwangerschapskans met SET te verhogen is meer informatie nodig over de criteria waaraan een embryo met een hoge implantatie kans moet voldoen. Naast de veel gebruikte criteria celaantal en embryomorfologie op de dag van terugplaatsing bleek het tijdstip van de eerste klievingsdeling een belangrijke factor (hoofdstuk 6). Embryo's die 23-26 uur na injectie (ICSI) of 25-28 uur na inseminatie (IVF) reeds uit twee cellen bestonden, implanteerden significant vaker dan embryo's die pas later kliefden. Het terugplaatsen van één vroeg gekliefd embryo resulteerde in een doorgaand zwangerschapspercentage van 37,1%, terwijl het terugplaatsen van één niet-vroeg gekliefd embryo maar in 10,3% van de patiënten in een zwangerschap resulteerde.

De vraag was echter of vroege klieving een onafhankelijke voorspeller voor zwangerschap was, of dat vroege klieving gerelateerd was aan celaantal en embryomorfologie op dag 2 en daardoor geen extra informatie kon verschaffen. Met behulp van een logistische regressie-analyse bleek dat

vroege klieving, naast celaantal en embryomorfologie, een onafhankelijke voorspeller was voor zowel zwangerschap als blastocystvorming.

Niet alleen uiterlijke kenmerken karakteriseren de kwaliteit van een embryo. Ook binnenin een eicel of embryo bevindt zich informatie die gerelateerd kan worden aan embryokwaliteit. In hoofdstuk 7 staat een onderzoek beschreven waarbij is gekeken naar de gen-expressie in cumuluscellen. Deze cellen omgeven de intra-folliculaire eicellen. Verschillen in intra-folliculaire condities die voor de eicel en latere embryo ontwikkeling van belang zijn, worden dus mogelijk weerspiegeld in verschillen in expressie van bepaalde genen in de cumuluscellen. Om deze verschillen op te sporen werd met behulp van microarrays naar de genoom-brede gen-expressie gekeken in cumuluscellen waarvan de eicellen uitgroeiden tot kwalitatief goede en minder goede embryo's. De expressie van

611 genen bleek gerelateerd te zijn aan embryo-ontwikkeling. Deze genen waren onder andere betrokken bij de celcyclus, angiogenese, apoptose, groeifactorproductie en chemokine- en cytokinesignalering en hypoxie. Ook al is verder onderzoek nodig, deze resultaten duiden mogelijk op intra-folliculaire stress omstandigheden ten gevolge waarvan de eicellen uit zouden kunnen groeien tot embryo's met een verlaagde implantatiekans.

In hoofdstuk 8 worden de resultaten bediscussieerd en wordt de mening van verschillende actoren die betrokken zijn bij het opstellen van een terugplaatsbeleid bij IVF, zoals clinici, patiënten, embryologen, politici en gezondheidseconomen, besproken. Concluderend blijkt dat op dit moment een SET strategie niet voor alle IVF patiënten geschikt is, maar dat het alleen toegepast dient te worden in een geselecteerde groep. Wat die selectie criteria zijn, niet alleen de embryo selectie criteria maar ook de patiënt-selectiecriteria, dient nog verder onderzocht te worden.

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En tenslotte natuurlijk pap en mam. Volgens mij staat er in dit boekje voor jullie niets nieuws meer. Jullie hadden zoveel oprechte interesse voor m'n werk, af en toe zelfs misschien een beetje te veel. En altijd stonden jullie klaar om naar m'n klaagzang te luisteren als het in mijn ogen weer eens tegen zat. Ook hoe trots jullie op mij zijn mag iedereen (helaas) weten. Toch geeft het wel aan hoeveel vertrouwen jullie in mij hebben. Bedankt pap en mam, voor alles, ik hou van jullie en een dikke kus!

Curriculum Vitae

Aafke van Montfoort werd op 2 oktober 1979 geboren te Weert. In 1997 behaalde zij haar VWO-diploma aan de Scholengemeenschap Bisschoppelijk College te Weert. In datzelfde jaar begon zij aan de Universiteit Maastricht met de studie Gezondheidswetenschappen, met als afstudeerrichting Biologische Gezondheidkunde. Tijdens haar afstudeerstage bij de afdeling Medische Microbiologie van het Academisch ziekenhuis Maastricht deed zij, onder leiding van dr. C. Vink en dr. J. Heemskerk, onderzoek naar de betrokkenheid van R33- en R78-eiwit bij calcium signalering in rat cytomegalovirus. Aansluitend deed zij een tweede stage bij de afdeling fysiologie aan de Universiteit van Cambridge (Engeland), onder leiding van Dr. S. Sage en Dr. J. Heemskerk. Hier onderzocht zij de regulatie van 'store mediated calcium entry' door glycoprotein VI en collageen-receptoren in bloedplaatjes. Eind 2001 behaalde zij haar doctoraal diploma, waarna zij vanaf begin 2002, als promovendus binnen het IVF laboratorium van het Academisch ziekenhuis Maastricht, de in dit proefschrift beschreven onderzoeken heeft gedaan. Het onderzoek werd uitgevoerd binnen de divisie Ontwikkelingsbiologie van het onderzoeksinstituut Groei en Ontwikkeling (GROW) en stond onder leiding van Dr. J.C.M. Dumoulin Prof. J.L.H. Evers en Prof. J.P.M. Geraedts. Sinds april 2007 voert zij binnen datzelfde IVF laboratorium onderzoek uit naar de invloed van in vitro fertilisatie (IVF) technieken op de X-inactivatie en imprinting-status van uit IVF voortkomende embryo's.

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